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HEAT STABLE ENZYMES FROM THERMOPHILES

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Alkaline phosphatase is widely used in the military and civilian sectors. Commercially available enzyme from calf intestine is the weak link in many applications, breaking down when exposed to elevated temperatures or after being stored for prolonged periods.

An extremely heat stable alkaline phosphatase produced by a thermophilic bacterium had been previously identified. The major deficiency was a low specific activity. Research described in this report focused on increasing the specific activity of the heat stable enzyme. This was successfully accomplished by cloning the alkaline phosphatase gene from the wild type into E. coli. One 40-kd cloned product was purified to near homogeneity by nickel affinity chromatography of the N-terminal histidine tagged protein. Purified, cloned alkaline phosphatase exhibited good activity and was thermally stable.

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SUMMARY

Alkaline phosphatase is one of the most widely used enzymes with numerous uses in both the military and civilian sectors. The commercially available enzyme from calf intestine breaks down when exposed to elevated temperatures or after prolonged storage. The alkaline phosphatase is often the weak link in many systems applications.

A thermophilic organism producing an extremely heat stable alkaline phosphatase had been previously identified. The single major deficiency was that the specific activity was about 5 orders of magnitude less than the calf intestine alkaline phosphatase. Potential customers were willing to accept a product with specific activity one order of magnitude below what they were currently using since the great thermal stability was such an important benefit. However, any lower activity was not acceptable.

The focus of the research described in this report was to increase the specific activity of the heat stable alkaline phosphatase. This was successfully accomplished by cloning the alkaline phosphatase gene from JKR209, the wild type, into *E. coli*. One 40 kd protein demonstrating thermally stable alkaline phosphatase activity was selected for further work. The cloned gene product from JF2 was purified to near homogeneity by nickel affinity chromatography of the N-terminal histidine tagged protein. The purified, cloned alkaline phosphatase exhibited activity within 1 to 2 orders of magnitude of calf intestine alkaline phosphatase and was far more thermally stable.

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PREFACE

The work described in this report was authorized under Contract No. DAAM01-95-C-0029. This work was started in March 1995 and completed in March 1997.

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HEAT STABLE ENZYMES FROM THERMOPHILES

INTRODUCTION

Alkaline phosphatase is one of the most widely used enzymes. In immunodetection, alkaline phosphatase generates a reproducible and readily detected signal. However, in many applications, the heat labile enzyme proves to be the weak link. During Phase I research, two thermophilic bacteria were isolated which produced a very heat stable alkaline phosphatase. The enzyme was exported from the cell, simplifying the processing procedure. However, the specific activity was several orders of magnitude less than the usual source of commercially available alkaline phosphatase, namely calf intestine. The main objective of the Phase II work was to increase the specific activity of this very stable alkaline phosphatase to make it a commercially viable product.

Naturally occurring bacteria seldom have all the attributes required for a commercially valuable organism. For mesophilic bacteria, one useful approach has been to manipulate the genetic information from a wild type organism, and clone it into a more readily handled organism with vastly improved production capacity. This has not yet become a routine procedure for thermophiles.

There were no reports of a successful transfer of alkaline phosphatase genes (e.g., *phoA*) from a thermophile into a more readily handled and more productive mesophile at the time this project was initiated. Cloning the alkaline phosphatase gene of a thermophile had been attempted numerous times; in some cases, gene transfer had been accomplished but the enzyme was not expressed. However, considerable structural information was available for several bacterial alkaline phosphatase genes including complete gene sequences and cloning procedures, vectors, hosts etc. used to clone the *phoA* gene in mesophilic organisms. One objective of this project was to clone the gene responsible for alkaline phosphatase production from the thermophile, JKR209, into an alternative, better producing organism.

CLONING OF ALKALINE PHOSPHATASE GENE AND PRODUCTION OF HIGH SPECIFIC ACTIVITY ENZYME

Cloning into *E. coli* and expression of high activity alkaline phosphatase:

Restriction enzyme digest fragments of JKR209 genomic DNA were ligated into an expression vector and transformed into competent *E. coli*. The library of alkaline phosphatase candidates were screened for alkaline phosphatase levels beyond endogenous levels. Electrophoretic analysis of expressed proteins from one candidate, JF2, demonstrated a 40 kilo dalton protein with thermally stable alkaline phosphatase activity. Based on the relative molecular weight of the wild type alkaline phosphatase from JKR209, it was estimated that this cloned gene product was lacking approximately 40 amino acids. Therefore, efforts were applied toward the identification and cloning of

the complete nucleotide sequence using the available clone as a probe. High stringency Southern analysis of fragmented JKR209 genomic DNA identified several larger fragments which may contain the complete sequence. No effort has yet been applied toward cloning these fragments. The cloned gene product from JF2 was purified to near homogeneity (approximately 90% pure) by nickel affinity chromatography of the N-terminal histidine tagged protein (Novagen). The relative enzymatic activity of the purified cloned alkaline phosphatase was found to be within 1-2 orders of magnitude of commercially available calf alkaline phosphatase. In addition, the cloned enzyme was more thermally stable than either the calf intestinal or commercially available bacterial alkaline phosphatase.

Preparation for cloning:

Genomic DNA was purified from a culture of JKR209 by standard methods and subsequently digested with the restriction enzyme Sau3A I. Restriction fragments ranging in size from 500 bp to 2000 bp in length were gel purified and the Sau3A I cohesive ends were ligated into the BamH I site of a pET 23A expression vector (Stratagene). The resulting plasmid DNA was transformed into BL21 competent cells (Novagen). Transformants were screened for alkaline phosphatase activity beyond endogenous levels upon induction of the plasmid expression system with IPTG. One candidate expressing unusually high alkaline phosphatase activity was isolated and labeled JF2. BamH I digest of JF2 plasmid DNA resulted in a 1100 bp insert suggesting the cloning of only a partial sequence of the alkaline phosphatase from JKR209. Partial cloning of JKR209 alkaline phosphatase sequence was supported by SDS-PAGE analysis of the JF2 expressed product. Whereas the JKR209 alkaline phosphatase was approximately 45 kd in size, the JF2 product was only approximately 40 kd. Despite the apparent lack of approximately 40 amino acids from the carboxy terminus of the cloned alkaline phosphatase as compared to the wild-type enzyme, the cloned product exhibited 90% of the wild-type activity.

Initial studies demonstrated that the activity per microgram of purified cloned enzyme was comparable to that of the native enzyme from JKR209. Also the alkaline phosphatase activity from both the native and cloned sources appeared to be within an order of magnitude of the activity from commercially available calf intestinal alkaline phosphatase.

Further efforts were applied toward the isolation and cloning the complete native nucleotide sequence from JKR209 which encodes for its alkaline phosphatase activity. The entire recombinant plasmid JF2 was nick translated in the presence of ^{32}P-d-ATP and used to probe southern transfers of restriction digested JKR209 genomic DNA. Despite experimentation with varying stringency conditions, numerous bands were homologous to the probe. This may be due to sequence homology between the JKR209 DNA and the pBR vector DNA. The insert from JF2 was gel purified for future labeling and to be used as a more stringent probe of the JKR209 DNA.

Purification on nickel column by binding of histidine tags:

Many pET vectors have the advantage of carrying a stretch of 6 or 10 consecutive histidine residues that can be expressed at the N-terminal or the C-terminal end of the target protein. This histidine tag sequence binds to divalent nickel ions immobilized on a metal chelation resin. After the unbound proteins are washed away, the target protein is recovered by elution with imidazole. This versatile system provides a convenient, economical means of purification without the need to develop new protocols for each protein.

The *E. coli* with the cloned alkaline phosphatase gene was grown in LB medium. This enzyme carried six histidine residues at the N-terminus. Cells were lysed by sonication and the debris separated out by centrifugation. The soluble fraction was applied to the nickel column diluted in a sodium chloride buffer, added to improve binding of the enzyme with the histidine tag. The column was washed with buffer. It was then washed with a 40 mM solution of imadazole to remove materials that had bound nonspecifically. The tagged alkaline phosphatase was eluted from the column with a 4 M imadazole solution. The eluted material was dialyzed to remove the imadazole, leaving the largely purified alkaline phosphatase.

MOLECULAR BIOLOGY

The work described in the previous section was performed near the end of this project. The remainder of this report is a discussion of all of the work that led up to this successful conclusion. It was necessary to learn a lot about the alkaline phosphatase gene and about the wild type organism, JKR209, before the cloning and expression were performed.

Trypsin digest method used in protein sequencing of critical portions:

Partial sequencing of the heat stable alkaline phosphatase was done following a trypsin digest. 100 ρ moles of protein was mixed with 0.1 M ammonium bicarbonate (pH 8.0). A 10% trypsin stock in 0.1% TFA was used. The protein solution was incubated at 30 °C for 18 hrs. At time zero and again at 5 hrs, 4% trypsin was added. The digestion was stopped with 4 microliters TFA. The digested fractions were separated on a C-8 column by reverse phase HPLC.

Gene cloning techniques:

<u>Oligonucleotide probe designed from N-terminal sequence:</u>

A 17-mer was synthesized. Since the codon usage in JKR209 was not known, a degenerate probe was made. A genomic blot of JKR209 DNA was prepared. The

probe was labeled with Fluor-12-dUTP or with cloned T4 polynucleotide kinase and gamma ^{32}P-ATP. The hybridization temperature was either 27 or 32 °C. Low stringency washes were used.

A 29-mer was also synthesized. This probe was labeled with the cloned T4 polynucleotide kinase and gamma ^{32}P-ATP.

Oligonucleotide probes based on the internal sequence:

A 17-mer degenerate probe was synthesized. The 3' end of the probe was labeled using a nonradioactive labeling kit. Hybridization to the genomic blot was done at 45 °C. Low stringency washes were used.

PCR:

The hot-start, touch-down PCR method was performed. The reaction mixture contained 100 ng of template JKR209 genomic DNA, 100 ρ mol of primers (internal and N-terminal probes), and 1 unit of *Taq* polymerase. The *Taq* polymerase was added after the reaction tubes were incubated at 95 °C for 5 min. The annealing temperature was decreased in 0.5 °C increments at every cycle, from 67 to 57 °C for 20 cycles.

E. coli phoA gene:

E. coli S17-1 lambda pir, harboring a pUT plasmid (approximately 7.9 kb) with a *phoA*-kanamycin resistance insert, was grown in 5 ml LB broth with ampicillin and kanamycin. The plasmid was isolated and purified from this culture and digested first with BamH1 and then with HindIII to obtain the *phoA* insert of approximately 1.5 kb. The insert was randomly labeled with alpha ^{32}P-dCTP by the Klenow fragment of DNA polymerase I. Labeled probe was hybridized to a genomic blot of JKR209 at 45 °C. Low stringency washes were performed and the blot was exposed to film at -70 °C until developed.

Protein sequencing results and discussion:

Seventeen amino acids were sequenced from the N-terminal end of the purified alkaline phosphatase protein by the microsequencing laboratory at Washington State University. Table 1 lists the amino acid sequences determined for purified alkaline phosphatase and corresponding oligonucleotide sequences used in the study.

Table 1: Amino acid and oligonucleotide sequences for JKR209 N-terminal

N-terminal	17-mer N-terminal	29-mer N-terminal	Internal sequence 1	17-mer internal	Internal sequence 2
ser			val (ile)		ala (?)
lys			asp (asp)		ile/leu
pro			val (val)		ser (?)
ser			ile (ile)		gly
glu	5' GAR	5'GAR	leu (leu)		glu
val	GTN	GTN	gly (gly)	5' GGN	asp
lys	AAR	AAR	gly (gly)	GGN	gly
asn	AAY	AAY	gly (gly)	GGN	pro
val	GTN	GTN	glu (arg)	GAR	phe
val	GT 3'	GTN	asp (lys)	GAY	pro
leu		YTN	tyr (tyr)	TA 3'	ile
phe		TTY	x (met)		ala
val		GTN	tyr (phe)		arg
gly		GG 3'	pro (pro)		
asp (?)			ala (met)		
met			gln (gly)		
			asn (thr)		
			pro (pro)		
			x (asp)		
			tyr (tyr)		

The human alkaline phosphatase amino acid sequence is shown in parentheses for internal sequence 1.

Internal protein sequences were obtained by trypsin digests of the purified alkaline phosphatase sample. The digested fractions were separated on a C-8 column and sequenced. Four fractions were collected for sequencing. Two of these fractions could be sequenced (Table 1), and one matched the sequence of human alkaline phosphatase very closely.

Initial attempts to clone JKR209 alkaline phosphatase gene:

The first approach taken was to synthesize a degenerate probe of 17 oligonucleotides based on the N-terminal sequence (Table 1). Non-radioactive labeling and radioisotope labeling procedures were used to end-label the probe. Low stringency conditions were used to probe genomic blots containing JKR209 DNA digested with different restriction endonucleases. The subsequent washes were also of low stringency. The resulting autoradiographs showed nonspecific binding. A longer probe (29 oligonucleotides) was then synthesized (Table 1) and used to probe a genomic DNA blot. It was hypothesized that the longer length of the probe might compensate for the high degeneracy by conferring greater specificity. However, as with the shorter probe, unspecific binding occurred.

An oligonucleotide probe based on the internal sequence closely matching the human alkaline phosphatase sequence was then synthesized (Table 1). This was end-labeled with a non-radioactive labeling kit. Again, no strong signals were observed upon hybridization with a genomic DNA blot. Only the undigested, high molecular weight DNA hybridized with the probe.

The major difficulty in using the above approaches to clone the alkaline phosphatase gene was that the gene probes, synthesized on the amino acid sequences of the enzyme, were highly degenerate. This automatically lowered the specificity of the probe. Blotting and washing conditions had to be of low stringency, thus increasing the possibility of unspecific binding. It was known that using a larger DNA fragment (greater than 1 kb) would increase the chances of obtaining binding of probe to target DNA.

With this in mind, two approaches were taken to obtain large DNA fragments to probe for the alkaline phosphatase gene from JKR209. The first approach was to use the N-terminal degenerate oligonucleotide and the internal degenerate oligonucleotide in opposite orientation to amplify the alkaline phosphatase gene in a polymerase chain reaction (PCR). Since degenerate probes were used, the PCR protocol was performed so as to minimize unspecific amplification. This included adding the *Taq* polymerase after the template DNA had been fully denatured at 95 °C, and lowering the annealing temperature from 67 °C in small increments during the cycling to a final temperature of 57 °C. This procedure resulted in several PCR products appearing, of which one band was very intense, when viewed under UV light after staining the agarose gel with ethidium bromide. Since it was not readily apparent whether this band, or any of the others, was the amplified alkaline phosphatase gene, this approach was aborted.

The second approach was to use the *E. coli phoA* gene as a probe. This gene was isolated from a plasmid vector. The purified DNA fragment was approximately 1.5 kb in length and was randomly labeled with Klenow reagent. The probe was hybridized to a Southern blot of JKR209 DNA, containing an EcoRI-digested DNA of *E. coli* BW545 DNA. No hybridization signals were observed, even with the *E. coli* control. It was later determined that the *E. coli* BW545 has a lac deletion which may have extended into the alkaline phosphatase gene (these two genes are located next to each other on the *E. coli* genomic map). There may be little sequence similarity between the *E. coli* alkaline phosphatase gene and the JKR209 alkaline phosphatase gene, and this must be unambiguously determined.

An *E. coli* clone containing the *phoA* gene from *Lysobacter enzymogenes* was obtained from the University of Alberta. Like JKR209, this organism secretes alkaline phosphatase. Attempts to purify the plasmid from the *E. coli* strain were unsuccessful.

Fresh genomic DNA from *E. coli* C600 was isolated. This was digested with EcoRI, electrophoresed and blotted on a nylon membrane. A 1.6 kb DNA fragment encoding the *E. coli* alkaline phosphatase was purified and used to probe the blot. A 22 hr exposure of the blot indicated a weak hybridization signal of the probe with *E. coli* C600 (run as a positive control). The blot was re-exposed for six days to enhance the hybridization signal of *E. coli* C600. After that time, a 1.5 kb fragment of JKR209 showed a weak hybridization signal with the *phoA* probe.

Also, additional cells of JKR209 were produced and genomic DNA was isolated. This preparation was also digested with EcoRI and run on the same gel, blotted and probed with *phoA* gene from *E. coli* cloned in pUT. These blots were probed with a mixture of the degenerate primers synthesized for the alkaline phosphatase gene of JKR209.

Another gel was run with genomic digests of JKR209 and *E. coli* and a Southern blot was performed using a 29-mer oligonucleotide probe synthesized for the alkaline phosphatase gene of JKR209. The probe was labeled by following the rad-free system from Schleicher and Schuell. After a 15 hr exposure of the blot, only background signal was observed.

Parts of the 16S rDNA of JKR209 was amplified by PCR using standard 16S rDNA primers. An attempt to clone the PCR amplified products using the TA cloning kit was unsuccessful. In another approach, the PCR products were gel purified to be cloned into the SmaI site of Bluescript SK+. For this purpose, Bluescript SK+ was digested with SmaI, and also dephosphorylated.

A high concentration of genomic DNA of JKR209 was cut with EcoRI. This digest along with EcoRI digested *E. coli* C600 and *phoA* gene were blotted onto a nylon membrane. Three identical blots with the above mentioned samples were individually probed with 17-mer, 29-mer and 17+29-mer (mixture) oligonucleotide probes labeled with radioactive ^{32}P. The blots were first washed at room temperature for half an hour

with 6X SSC and washed again at 40 °C for half and hour with 6X SSC. The blots were exposed and the film was developed after 32 hrs. In the blot probed with the 17mer, a distinct band of hybridization with JKR209 was observed, although it is in the higher molecular weight range. Hybridization signals with JKR209 (higher molecular weight smear) were also noticed in the blots probed with 29-mer and mixture of oligonucleotide (17+29-mer) probes.

16S rDNA of JKR209 was amplified by PCR using universal primers. The amplified product (400 bp) was cloned using Clone Amp kit (Gibco, BRL).

Library construction: A library of JKR209 was constructed. Genomic DNA of JKR209 was digested with MboI so as to yield higher molecular weight fragments (>30 kb). MboI partially digested and alkaline phosphatase treated JKR209 genomic DNA was ligated into the digested vector pKC876. The ligated DNA was subsequently packaged into lambda using the Gigapack Gold II packaging kit (Stratagene). This experiment was repeated twice and only 135 clones were obtained, which only partially represents the JKR209 genomic DNA.

LB medium for clones and *E. coli* was as follows (gm/l):

Tryptone	10
Yeast extract	5
Sodium chloride	10
Agar	15

Ampicillin	0.050
Kanamycin	0.050

Ampicillin and kanamycin were for the *E. coli* raised at 37 °C.

Geneticin	0.020

Geneticin was for the library clones grown at 30 °C. The *E. coli* replicon is unstable above 35 °C.

Sequencing: Three independent 16S rDNA clones of JKR209 (#1, 4 and 6) were isolated. Plasmid DNA was isolated from these clones and PEG purified. This DNA was used for nucleotide sequence determination. The Sequitherm Long Read sequencing kit (Li-COR) was used to prepare samples for automated sequencing. Four hundred twenty four nucleotides were determined for each clone using forward and reverse primers. All three clones were identical and all ambiguities were resolved using the sequencing data of both strands. The sequence data was subjected to the "Fast Scan" similarity search program of PC Gene. The results of the search identified *Bacillus infernus* 16S rRNA gene as most similar to JKR209 with 397 bases out of 424 being identical. In order to determine the complete 16S rRNA gene of JKR209, the *Bacillus infernus* sequence was then used to design 16S rDNA PCR primers.

Four sets (1L 1R, 2L 2R, 3L 3R and 4L 4R) of 16S rRNA primers that were designed by Dr. Dave Knaebel were used to amplify the 16S rDNA of JKR209. The amplified product was then ligated with pGEM-T vector (Promega) and transformed into DH5α competent cells. Three representative clones from each set of the transformants were chosen and plasmid DNA was isolated for sequencing. Sequencing reactions were performed using Sequitherm kit (Epicenter Technologies) for automated sequencing.

Nucleotide sequence data were obtained from one representative clone from each set of the transformants. This sequence data was aligned and resulted in 734 bp of continuous sequence. A "Fast Scan" similarity search program of PC Gene identified *Bacillus infernus* and *Bacillus thermoalkalophilus* 16S rRNA gene as most similar to JKR209 with 664 and 676 bases out of 734 being identical. Sequence analysis of further clones will be necessary to clear up some existing ambiguities and to eventually obtain more than 1000 bases (unofficial requirement) necessary for better classification.

Further nucleotide sequence data was obtained and added to the existing JKR209 16S rRNA gene sequence. This sequence data was aligned and resulted in 1083 bp of continuous sequence. A "Fast Scan" similarity search in the ribosomal database project (RDP) identified *Bacillus caldotenax* 16S rRNA gene as most similar to JKR209.

UV radiation treatment:

To facilitate a search for overproducing mutants, JKR209 was incubated for 4 hrs and then exposed to short wave UV radiation for 7, 15 and 30 min. Cells were then incubated at 65 °C. The 30 min treatment killed all the cells. The colonies growing on the edge of the 7 and 15 min plates was used to inoculate another set of plates. It should be noted that allowing cells to grow for 24 hrs before UV radiation resulted in much more growth than was obtained in this experiment.

Four more sets of plates were prepared, exposing the plates to UV radiation for 3-6 min, allowing them to grow for 24 hrs, and then transferring colonies to a fresh set of plates. By the end of this treatment, most organisms had been killed, but two colonies were recovered. These two colonies were used to inoculate liquid cultures. These cultures were allowed to grow at 65 °C for two days. Alkaline phosphatase levels were determined in duplicate and reported as averages in the following table.

Table 2: Alkaline phosphatase produced by JKR209 and 2 UV irradiated mutants

Time (hr)	JKR209	Mutant A	Mutant B
20	0.220	0.172	0.215
32	0.334	0.259	0.328
48	0.586	0.468	0.546
68	1.175	0.877	1.335
corrected for volume change	1.000	0.695	1.515

There was a significant loss in volume because of the unusually long fermentation time. Correcting the alkaline phosphatase for the change, did not actually change the conclusions. Mutant A produced less enzyme throughout the fermentation, regardless of the volume correction. Mutant B produced roughly the same amount of alkaline phosphatase as the wild type, although it could be considered a slightly better producer when the volume change was taken into account. Mutant B was not such a superior producer to change organisms at this point.

Electrophoretic method:

Following are the detailed electrophoresis procedures as they were performed at J. K. Research. A similar method was utilized at the University of Idaho; this procedure is only related in detail once.

4X separating gel buffer

	gm/500 ml
1.5 M Tris base, pH to 8.8 with HCl	91.0
2% SDS	2.0

Whatman filter and store at 4 °C

4X stacking gel buffer

	gm/100 ml
0.5 M Tris base, pH to 6.8 with HCl	6.1
0.4% SDS	0.4

Whatman filter and store at 4 °C

10X running buffer

	gm/liter
Tris base	30.25
glycine	144.00
SDS	10.00

pH to 8.8 with HCl

Sample buffer

0.0625 M Tris base, pH to 6.8 with HCL	1.25 ml of 1 M Tris base
2% SDS	4.0 ml of 10% SDS
10% glycerol	2.0 ml of 100% glycerol
0.001% Bromphenol blue	20 microliters of 1% stain
5% β-mercaptoethanol	1.0 ml of 100% β-mercaptoethanol
water	1.75 ml

Store in 1 ml aliquots at -20 °C

30% Acrylamide (29.2% acrylamide, 0.8% bisacrylamide)

Dissolve 29.2 gms acrylamide in about 50 ml water
Add 0.8 gms bisacrylamide and dissolve
Bring total volume to 100 ml
Whatman filter and store at 4 °C in amber glass
Note: acrylamides are neurotoxins; wear gloves

10% APS

Dissolve 1 gm ammonium persulfate in 10 ml water
Store at 4 °C in amber glass up to 2 weeks

2% agarose in 0.375 M Tris base, pH 8.8

4.84 gms Tris base
pH to 8.8 with HCl
Add 2 gms agarose
Add 100 ml water
Microwave until dissolved
Store at approximately 45 °C in sealed bottle

10 ml 12% acrylamide separating (resolving) gel. (Sufficient for 2-3 small gels).

3.4 ml water
4.0 ml 30% acrylamide
2.5 ml 4X separating gel buffer
100 microliters 10% APS
10 microliters TEMED

5 ml 5% stacking gel

2.9 ml water
833 microliters 30% acrylamide
1.25 ml 4X stacking gel buffer
50 microliters 10% APS
5 microliters TEMED

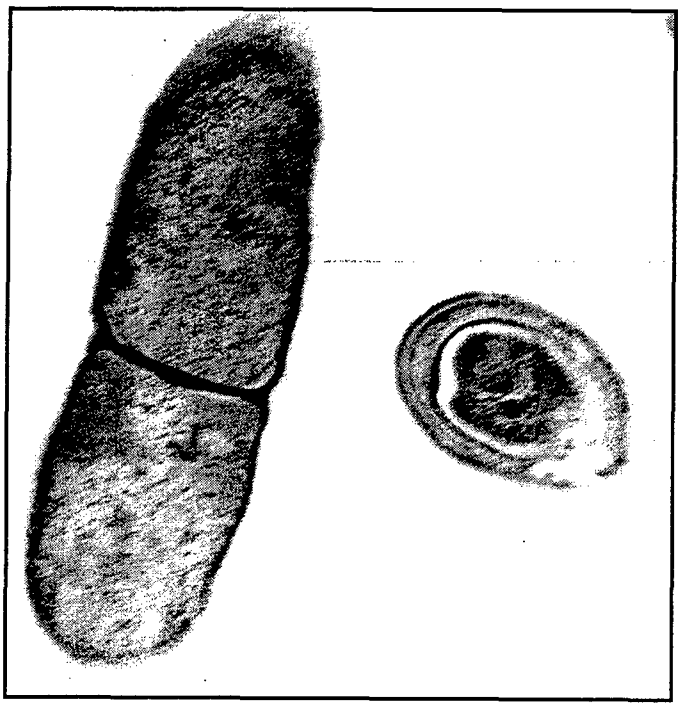
Assemble the glass plates and spacers, seal and clamp. Prepare resolving gel, adding the TEMED last. Pour immediately to 2 cm from top of plate. Overlay with water saturated butanol. Allow 1 hr for complete polymerization. Rinse off the butanol. Pour in the stacking gel and immediately insert combs. Allow 30 min for complete polymerization.

Dilute samples 1:1 with sample buffer, boil 2-5 min, cool, centrifuge and apply to gel. Electrophorese at 75 volts until samples reach the resolving gel. Voltage may then be increased up to 150 volts. Stain overnight in 0.1% Coomassie blue R250, 20% methanol, 10% acetic acid. Microwaving for 30 sec will speed the staining process as will addition of 0.1% cupric acetate. Destain in 20% methanol, 10% acetic acid, adding fresh solution every 10 min. Store in distilled water.

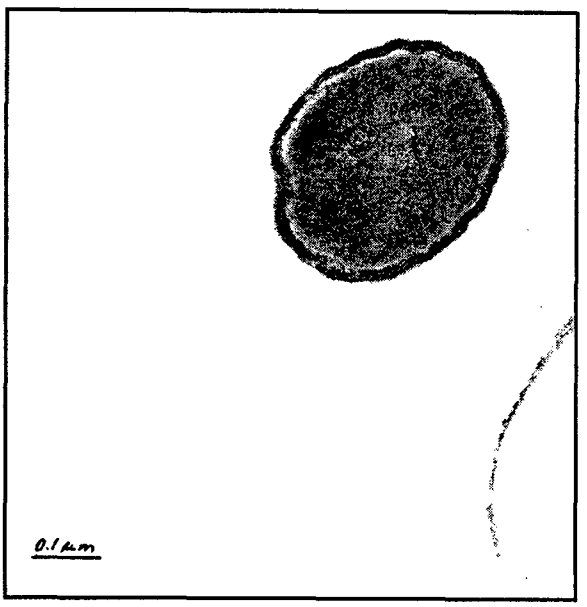
IDENTIFICATION OF JKR209

JKR209 was cultured in BUGM (Biolog universal growth medium) and BUGM + 1% glucose plates. 14 hr cultures were washed in 0.85% saline solution, and used to inoculate gram positive Biolog plates. The plates were incubated at 55 °C overnight. Comparison of the Biolog data in the database (3.7 version) did not yield any clear identification; the closest species was *Bacillus thermoglucosidasius* with a similarity index of 0.35.

Electron micrographs (TEM) indicated that JKR209 is a rod shaped bacterium. Presence of spores were presumably evident from the micrographs.



External longitudinal view and lateral cross-section of JKR209.



View of a spore of JKR209.

ALKALINE PHOSPHATASE ASSAY

Assay pH:

Diethanolamine was adjusted to pHs between 9.6 and 11.0 with hydrochloric acid. The experiment was repeated twice. Column A shows the results from a single set of assays; column B is the average alkaline phosphatase level determined from 2-4 assays per pH unit tested. Th optimum pH was between 9.8 and 10.0.

Table 3: Effect of assay pH on alkaline phosphatase activity

pH	A	B
9.6	0.512	0.405
9.8	0.512	0.424
10.0	0.552	0.415
10.2	0.462	0.342
10.4	0.424	0.286
10.6	0.361	0.268
10.8	0.312	0.223
11.0	0.257	0.200

Concentration of Tris:

Both Tris and diethanolamine are phosphate acceptors so are found in most methods for alkaline phosphatase assays. Tris was prepared at a range of concentrations. Assays were performed in triplicates. There was a minimal increase in activity as the Tris concentration was increased, but statistically insignificant. Several standard methods use 20 mM Tris, so this was the value used for the remainder of the experiments.

Table 4: Effect of Tris concentration on alkaline phosphatase activity

mM Tris	Average alkaline phosphatase
10	0.527
20	0.530
40	0.531
100	0.533

Effect of metals on alkaline phosphatase activity:

Solutions of metal salts were prepared; final concentrations in the assay medium were 2, 5 and 10 mM calcium chloride, 1 mM magnesium chloride, 0.5 mM manganese chloride, 0.5 mM ferric citrate and 1 mM zinc sulfate. These were added to the assay tubes in all possible combinations.

When interpreting these results, it must be remembered that the alkaline phosphatase was produced in a medium where all these elements were present although the levels of some were extremely low. Although the enzyme was largely purified, these metals could be bound very tightly at the active site. This was not an experiment to determine which metals were most useful at the active site, but rather which could be added under use conditions to maximize the activity.

Analysis of one alkaline phosphatase concentrate, following EPA 200.2 digestion, revealed the presence of low levels of zinc, manganese and magnesium with higher levels of iron and calcium as shown in Table 5.

Table 5: Metals analysis of alkaline phosphatase concentrate

Metal	mg/l
Cobalt	<0.01
Manganese	0.04
Zinc	2.3
Magnesium	9.0
Iron	24.6
Calcium	1820.

Addition of calcium chloride was the most beneficial. This was a little surprising because there is so much calcium chloride in the growth medium and in the elution buffer used during purification and the analysis of the concentrate revealed the presence of quite high levels. It was anticipated that additional calcium was not necessary, but this proved to be untrue.

Another unanticipated result was that some elements actually had a negative effect on the alkaline phosphatase activity. Zinc was not of any apparent value under any circumstances, although it has been found a useful addition to the production medium. A low level of zinc was found bound to the enzyme.

In the following table, no elements were added to the control tube. Diethanolamine and Tris buffers were prepared without addition of any test elements. Test elements were added separately to each tube. The same dilution of alkaline phosphatase was used for all assays, allowing comparisons to be made. In combinations, 5 mM calcium was used. Assays were performed in triplicate and the average was recorded here.

Table 6: Effect of metals on alkaline phosphatase activity

Metals added	Alkaline phosphatase
Control	.112
Mn + Zn	.047
Mg + Zn	.050
Mg + Mn	.051
Fe + Zn	.054
Zn	.056
Mg + Mn + Zn	.062
Mn	.064
Fe + Mg + Zn	.066
Fe + Mn + Zn	.067
Fe + Mg + Mn + Zn	.069
Fe + Mn	.071
Ca + Mg + Mn + Zn	.081
Fe + Mg + Mn	.083
Ca + Mn + Zn	.088
Mg	.093
Ca + Fe + Mn + Zn	.093
Ca + Fe + Zn	.106
Fe	.118
Ca + Zn	.119
Ca + Mg + Zn	.134
Ca + Fe + Mg + Zn	.149
Ca + Mg + Mn	.158
Ca + Mn	.161
Ca + Fe + Mg	.171
Fe + Mg	.175
Ca + Fe + Mg + Mn	.218
10 mM Ca	.276
5 mM Ca	.280
Ca + Mg	.284
2 mM Ca	.301
Ca + Fe + Mg	.310
Ca + Fe	.320

Effect of mannitol on assay:

Mannitol is a phosphate acceptor, and as such, would be expected to increase alkaline phosphatase activity, in much the same manner as diethanolamine. However, mannitol did not have a beneficial effect when added to the assay tubes at a rate of 0.2 gm/tube.

Control	19.300
0.2 gm mannitol/tube	17.500 (average of two separate runs)

Optimized alkaline phosphatase assay:

Following is the optimized assay method. Experiments used to develop this method are discussed below.

Diethanolamine reagent:

105.08 diethanolamine
0.20 magnesium chloride
894.72 water
pH to 9.9 with concentrated HCl

Substrate:

1 capsule (40 mg) pNPP in 110 ml diethanolamine reagent
or
1 tablet pNPP (20 mg) in 55 ml diethanolamine reagent

Assay buffer:

20 mM Tris
0.5 mM ferric citrate
5 mM calcium chloride
pH to 8.6 with 1 N HCl
Ferric citrate dissolves slowly; allow to stand overnight before use.

Assay procedure:

2 ml buffer
50 µl fermentation broth
2 ml diethanolamine reagent with substrate pNPP
Time with a stop watch.
Read at 405 nm after 5 min at room temperature.

PRODUCTION OF ALKALINE PHOSPHATASE

Inoculum production:

Although phosphate is required for biomass production, the presence of too much phosphate is inhibitory to the production of alkaline phosphatase, due to feedback inhibition. Without sufficient biomass production, alkaline phosphatase levels will be low. Two approaches to overcoming this problem were tested. In both cases, the biomass was produced in a medium enriched with phosphate.

1. Add a phosphate acceptor to tie up the phosphate, forcing alkaline phosphatase production.

2. Transfer the cells from the enriched medium into a second medium, low in phosphate to force alkaline phosphatase production.

Option 1: Allophosite was tested as described more fully under alkaline phosphatase production medium, other components section. This was not successful.

Option 2: A variety of enriched media were tested for the ability to produce large amounts of cells. In the following table, the biomass was tracked as absorbance at 600 nm. Alkaline phosphatase is in arbitrary units. The treatments were then ranked according to their ability to produce a large amount of biomass and according to the alkaline phosphatase production. After 52 hrs in the rich media, fermentations were taken down and centrifuged at 3000 rpm for 40 min in 500 ml sterile jars. Cells were washed twice with 50 ml sterile, distilled water and centrifuged in sterile 50 ml tubes.

The formula that provided the best overall results in this experiment, considering both biomass and alkaline phosphatase production was 5 gm/l yeast extract, 5 gm/l proteose peptone and 5 gm/l malt extract.

Table 7: Inoculum formulation

Media components for inoculum gm/l	Abs_{600}	AP	pH	Rank Abs	Rank AP
5 yeast extract, 5 proteose peptone, 5 malt extract	1.015	.312	5.76	1	2
5 proteose peptone, 1 ammonium sulfate, 1 sodium acetate	0.506	.351	8.70	6	1
20 dextrose, 0.5 ammonium sulfate, 0.1 sodium nitrate, 1 tryptone	0.500	.300	5.19	7	4
20 sucrose, 0.5 ammonium sulfate, 0.1 sodium nitrate, 1 tryptone	0.646	.254	7.03	3	6
20 molasses, 0.5 ammonium sulfate, 0.1 sodium nitrate, 1 tryptone	0.338	.033	7.35	8	8
5 yeast extract	0.195	------	9.46	9	NA
5 proteose peptone, 10 dextrose, 10 sucrose	0.522	.274	5.23	5	5
5 yeast extract, 10 dextrose, 10 sucrose	0.686	.114	5.39	2	7
1 yeast extract, 1 peptone, 1 dextrose, 0.5 malt extract	0.542	.308	9.15	4	3

Enzyme production experiments have always indicated that yeast extract is inhibitory. This despite the fact that, of the components normally analyzed, there is little difference between yeast extract and alternative sources of nitrogen. Further experiments were done attempting to find suitable substitutes for the yeast extract. Calcium chloride was added at very low levels; it is known to inhibit phosphate uptake. Although this was not the goal during biomass production, the presence of a low level in the inoculation medium did seem to be of some benefit later during enzyme production. A stock formulation of 5 gm/l malt extract, 5 gm/l dextrose, 0.1 gm/l calcium chloride, and 1 gm/l trace elements was established.

Further work testing various nitrogen sources, indicated that tryptone was useful, although proteose peptone could be substituted almost as effectively. As the formulation was optimized it was found that the best formulas for producing the largest amount of biomass, later also resulted in production of the most alkaline phosphatase, possibly simply because there were more cells present initially, or perhaps certain metabolic pathways had been stimulated.

Alkaline phosphatase production medium:

Minifermenters proved to be very useful, particularly for determining the effects of changes in the media components. Up to 40 minifermenters could be run at a time,

providing sufficient data to make reliable decisions. However, alkaline phosphatase production was never as high in the minifermenters as they were in the New Brunswick type, larger, stirred fermenters. This may have been related to better control over the oxygenation or to more even mixing in the larger volumes.

At the beginning of the Phase II work, the maximum alkaline phosphatase produced in a set of experiments was 4.58/ml. By the end of the project, optimization of various parameters allowed alkaline phosphatase production to reach 25.4/ml, over a 5.5 fold increase for the native enzyme. Because of this general rise in values over the course of the work reported here, results in this section are reported as a percentage of the highest value for a particular run rather than trying to compare absolute values over the course of the entire project.

Absorbance is not an accurate measure of cell production because in some fermentors the cells stuck to the walls of the reactor or clumped together, but this observed phenomenon varied among reactors in no apparent pattern.

Medium from the literature: There are numerous references detailing media for alkaline phosphatase production. Selected for trial here was the formulation for a medium used by de Prada et. al. for alkaline phosphatase production in psychrophiles. It was adapted and set up in a 500 ml minifermenter as follows:

0.06 M Tris base, adjust with Tris HCl to 8
5 gm/l peptone
2 gm/l glucose
0.2 mM calcium chloride
0.23 gm/l NaCl
0.07 gm/l KCl
0.02 M ammonium chloride
1 mM magnesium sulfate
0.004 mM zinc chloride
0.016 gm/l sodium phosphate dibasic
pH salts to 8.1 with Tris HCl in 60% of the water, autoclave peptone and glucose in 40% of the water.

At 24 hrs, the alkaline phosphatase was 2.56 per ml, which was very low. No further attempt was made to use this medium which had worked well for the production of alkaline phosphatase by another organism.

Tables for results of testing carbon source, nitrogen source, trace elements and other media components are summaries from 11 separate runs. In most cases, tabulated results are averages of duplicate fermentations.

Carbon source: It has been demonstrated by others and during the Phase I work of the present project, that the carbon source can have a significant impact on alkaline

phosphatase production. (Nahas) However, this can be a rather complicated relationship. For example, one study found that the replacement of sucrose with acetate would increase alkaline phosphatase production unless the medium was buffered. In the later case, sucrose resulted in production of higher enzyme levels than did acetate. (Nahas)

Molasses contributes to the color of the final product and is somewhat messy to work with. Therefore, alternatives were tested. However, no combination of corn syrup, alternative sugars and vitamins was ever found to replace molasses. During Phase I, out of 11 carbon sources tested, molasses consistently gave superior results. After numerous trials here, molasses still resulted in production of the highest levels of alkaline phosphatase. During Phase I, it was found that replacement of molasses with sucrose, the major sugar in molasses, resulted in decreased production. However, when combined with the molasses, it has now been established that sucrose enhances target enzyme production.

When reviewing the next three tables, it must be kept in mind that the base formulation was changed as components were found to be useful or inhibitory. Also, sometimes when certain elements were being tested, other elements were eliminated from the formulation that may have been necessary, leaving a false impression that the element listed was inhibitory, rather than just having no effect. It was not possible to put all of this information into tabular form, but the interpretation of the results was made with this additional information in mind. This was particularly true of the trace elements but was of relatively little importance in the carbon source experiments.

Cells were centrifuged at 3200 rpm for 35 min and drained. They were then transferred to the minimal alkaline phosphatase medium with the following additions (in gm/l):

Table 8: Testing of potential carbon sources

Treatment	pH	Alkaline phosphatase per ml	% Maximum
10 sucrose	7.08	17.74	100
10 maltose	7.40	13.08	100
10 sucrose	7.72	11.32	100
2 trehalose	7.59	11.28	100
5 sucrose		10.64	100
10 sucrose + 5 mannitol	7.49	12.68	97
10 glycerol	7.33	12.26	93
10 sucrose	7.54	12.14	93
10 glycerol	6.05	15.94	90
10 glucose	7.17	11.80	90
10 sucrose, 2.5 mannitol 24, 30 & 46 hr	7.17	15.72	89
10 honey	5.22	11.44	87
Brer Rabbit molasses	7.66	11.36	87
5 sodium acetate	8.05	9.68	86
10 sucrose	7.14	11.18	85
5 mannitol	7.54	9.24	82
10 sucrose + 5 Na acetate	7.80	10.30	79
10 sucrose + 5 sorbitol	7.64	9.98	76
20 sucrose		7.80	73

Treatment	pH	Alkaline phosphatase per ml	% Maximum
10 honey, pH adjust at 24 and 33 hr	7.14	12.78	72
10 maltose	7.21	12.64	71
10 maltose		6.28	59
5 calcium acetate		3.68	35
10 dextrose	5.12	3.58	32
10 fructose		2.28	21
No molasses, tryptone, poly, vitamins	7.30	1.78	16
10 calcium acetate		1.36	13
10 arabinose		1.04	10

Maltose and trehalose were as effective as sucrose but are more expensive, an important consideration for commercial production.

Nitrogen source:

Table 9: Testing of potential nitrogen sources

Treatment	pH	Alkaline phosphatase per ml	% Maximum
2 NH_4SO_4, 1 KNO_3	5.70	18.20	100
2 KNO_3	7.57	10.76	95
5 NH_4SO_4, 1 KNO_3	5.14	14.88	82
5 KNO_3	7.61	10.08	81
1 NH_4SO_4, 1 KNO_3	6.08	14.64	80
1.0 malt	7.42	13.66	77
1 KNO_3	7.58	9.15	74
1.0 beef extract	7.58	12.80	72
1 NH_4SO_4	7.39	8.68	70
0.1 tryptone	7.43	11.70	66
1.0 vegetable peptone	7.79	10.92	62
4 NH_4SO_4	7.25	7.54	61
0.1 malt	7.43	8.52	48
0.1 casein	7.42	8.50	48
0 NH_4SO_4, 1 KNO_3	6.53	8.24	45
0.1 vegetable peptone	7.49	8.04	45
0.1 beef extract	7.56	7.96	45
1.0 casein	7.58	7.78	44

Treatment	pH	Alkaline phosphatase per ml	% Maximum
5 KNO_3, 1 NH_4SO_4	7.17	5.76	32
0 NH_4SO_4	7.78	2.76	24
1.0 tryptone	7.74	2.16	12
2 KNO_3, 1 NH_4SO_4	7.24	2.11	12
0 KNO_3, 1 NH_4SO_4	7.24	1.77	10
1 KNO_3, 1 NH_4SO_4	7.22	1.52	8

Trace elements:

During Phase I, manganese was found to significantly enhance alkaline phosphatase activity when added to the assay buffer. However, it did not enhance enzyme production when added to the medium, and, by the end of the testing when assay conditions and production media had been optimized, manganese was no longer of significant benefit in the assay buffer either.

Many of the trace elements tested either had no effect or were detrimental to production of alkaline phosphatase. The requirement for a high level of calcium had been previously established. It should be noted that the calcium requirement is not common among alkaline phosphatase producing organisms. In fact Goldman et. al. pointed out that the alkaline phosphatase produced by the halophile *Haloarcula marismortui* was unlike most alkaline phosphatases in its requirement for calcium. (Goldman)

Cobalt has been shown to result in production of 35 times more alkaline phosphatase as compared to a similar low phosphate medium without cobalt by *Bacillus licheniformis*. (Spencer) Cobalt chloride has been used by others to increase the excretion rate of alkaline phosphatase from the cell. When added after enzyme production had begun, it did not harm production. Eventually, however, it was dropped from the formulation since there was no compelling evidence of significant beneficial effects in the stirred reactors.

Ferric citrate added in combination with other elements was beneficial, although when added alone, less enzyme was produced. This appears to have been more a need for the other elements rather than an inhibitory effect by the ferric citrate itself.

Aluminum, copper, manganese, molybdenum and vanadium were not useful additions.

Table 10: Effect of trace elements on alkaline phosphatase production

Treatment	pH	Alkaline phosphatase per ml	% Maximum
.001 $AlSO_4$	7.23	12.78	72
.05 mM $CoCl_2$, 36 hrs	7.57	12.41	100
.05 mM $CoCl_2$, 24 & 36 hrs	7.52	12.14	98
.05 mM $CoCl_2$, 12 hrs	7.50	10.60	94
0.1 mM $CoCl_2$, 24 hrs	7.20	16.06	91
.002 $CuSO_4$	7.31	12.02	68
.02 Ferric citrate	7.42	17.70	100
.01 $MnSO_4$	7.43	11.02	62
.01 $MnSO_4$		2.20	21
.01 Na_2MoO_4	7.31	12.84	73
.01 Na_2MoO_4		1.60	15
.001 $VOSO_4$	7.57	10.36	84
.001 $VOSO_4$	7.26	13.24	75

Other components:

Even moderate levels of phosphate repress production of alkaline phosphatase. Since all organisms require a source of phosphorus for such critical products as ATP, media low in phosphorus inhibit biomass production. One way to work around this dilemma was to raise the organism in a medium not limited in phosphate. Then once cells were produced, addition of a phosphate acceptor would signal the organism that it has been switched to a low phosphate environment. The gene responsible for alkaline phosphatase synthesis should be derepressed and produce enzyme. For this study, allophosite, a hydrated alumina/silica gel, was added to serve as a phosphate trapping agent. Addition to enriched media had no effect, and as can be seen below, addition to the optimized alkaline phosphatase production medium was very inhibitory. It is

assumed that the allophosite trapped the very low levels of phosphorus that were present, thus essentially shutting down all enzyme production.

An antifoaming agent, Antifoam 289 from Sigma, was tested in the event that an antifoaming agent would be needed as the process was scaled up. There was minor foaming in the 5 and 14 liter fermenters, but not enough to require the use of an antifoaming agent.

Citric acid appears to be very inhibitory, but more likely, it was the effect on the pH that had a greater effect rather than the citrate.

Alkaline phosphatase does require metals at its active site. When the sequestering agent, EDTA, tied up available metals, the enzyme activity fell dramatically, as would be expected.

Envirofirst is sodium carbonate peroxyhydrate produced by Solvay. It is an inorganic peroxide and was added to provide an additional source of oxygen. 0.1 gm Envirofirst per liter exerted a minimally negative effect, but as the rate was doubled to 0.2 gm/l, the negative effect was more marked.

Ethanol has been used to stimulate alkaline phosphatase production.

Sodium and potassium chloride were tested because it is known that a low sodium, high calcium will result in phosphate deficiency inside cells, thus derepressing genes responsible for alkaline phosphatase production. As would be expected, addition of sodium was not useful. Potassium may work by a similar mechanism.

Tripolyphosphate was tested as an alternative source of phosphate. It was reasoned that a less readily accessible source of phosphate would stimulate alkaline phosphatase production, while still providing the phosphate needed for other cellular functions. However, tripolyphosphate did not prove to be useful.

Pyrroloquiniline quinone (PQQ) is found in a wide range of microorganisms. The existence of different classes of PQQ-containing enzymes is well established and includes certain dehydrogenases and oxidases. Several other classes of enzymes are suspected to be quinoproteins. Many bacteria produce a quinoprotein apoenzyme, requiring the addition of PQQ. (Duine)

Studying the ability of enteric bacteria to survive in harsh environments, Adamowicz et. al. found organisms that could grow on glucose only if they were provided with the coenzyme PQQ. However, PQQ appeared to have difficulty entering the periplasm except under low phosphate growth conditions. It is well documented that the *PhoE* porin would be expressed under these conditions. (Adamowicz) Thus it was reasoned that alkaline phosphatase could be produced as the apoenzyme, requiring PQQ for activation. PQQ is produced by many organisms and excreted into the environment,

providing the essential nutrient for other members of the community. Under laboratory conditions, trace amounts found on laboratory glassware after improper washing, have been sufficient to stimulate growth of certain organisms. (Ameyama) Other known sources of PQQ include yeast extract and peptone. In a complex medium such as that developed for JKR209, it is assumed that some PQQ would be present. Here we were simply looking for a further increase in production of active enzyme in the presence of excess PQQ.

Thiamine is involved in phosphate transport but appeared to have no beneficial effect when added to the production medium. The vitamins added were 0.0050 gm biotin, 0.0500 gm nicotinic acid, 0.0250 gm thiamine HCl in 50 ml water.

Table 11: Testing of additional components

2.5 allophosite at 24 & 33 hr	7.04	1.74	10
2 allophosite		0.72	7
1 antifoam	7.60	13.12	100
1 antifoam	7.24	14.40	81
10 citric acid	1.92	1.88	14
1 EDTA	7.35	1.76	16
0.1 Envirofirst, 26 hrs	7.86	10.62	94
0.1 Envirofirst	7.62	10.06	89
0.1 Envirofirst, 24 & 36 hrs	7.70	10.38	84
0.2 Envirofirst		4.26	40
5 ml ethanol, 26 hrs	7.79	6.04	53
5 ml ethanol		4.78	45
2 KCl	7.34	13.10	74
2 NaCl	7.26	13.12	74
.0037 (.01 mM) tripolyphosphate	7.84	2.00	18
PQQ	7.39	8.48	75
10 vitamins	7.25	16.34	92
2 vitamins	7.50	9.60	85
10 vitamins	7.56	10.30	83

Fermentation temperature:

Although the organism JKR209 would grow over a wide temperature range, the optimum temperature for alkaline phosphatase production was determined and the results are tabulated in the following table.

Table 12: Optimum fermentation temperature for alkaline phosphatase production

Temperature	Alkaline phosphatase/ml
69	0.074
	0.071
66	0.066
	0.163
62	0.351
	0.379
59	0.215
	0.528

Time course:

Numerous experiments were run in the 5 and 14 liter fermenters. The 5 liter fermenters were of particular value since there were three that were identical, permitting experimentation with some medium components at the same time that parameters most readily monitored in a stirred fermenter were being evaluated. Following are three examples of runs. All of the fermenters listed here were inoculated at a rate of 0.5%. 24 hr old cultures were centrifuged and the spent medium was drained. The cells were not washed.

Fermentations were run at 65 °C; aeration enhanced evaporation. Typically 40% of the original volume was lost if condensers were not used or if the volume was not replaced. Therefore, half salts were added as needed to maintain the volume. It had been determined earlier that replacing liquid lost to evaporation significantly enhanced enzyme production.

Run 1:

In this run, the fermenters were started at 100 rpm with 0.5 ml air/ml medium/min. At 2 hr, the aeration rate was increased to 1 ml air. At 4 hr, the agitation was increased to 300 rpm and then decreased back to 200 rpm at 36 hrs. The volume was maintained by addition of half salts starting at 8 hrs. After half salts were added, the fermenters

were allowed to mix for 5 min before samples were taken. During this run, at some time between 8 and 16 hrs, the agitator in B stopped, but was repaired and was back on at 16 hrs. The aeration rate was sufficient to aid in mixing, but the agitator helps to disperse the air bubbles. It is likely that dissolved oxygen levels decreased during the time when the agitator was not working.

The medium was as follows:

Salts formulation:

1 gm/l ammonium sulfate
5 gm/l calcium chloride
3 gm/l Tris base
Adjust to pH 8.1 with Tris HCl before autoclaving.

Molasses etc formulation:

0.1 gm/l tryptone
10 gm/l sucrose
0.02 gm/l ferric citrate
3.0 gm/l molasses

Metals formulation:

0.005 gm/l magnesium sulfate
0.001 gm/l zinc sulfate

Additional nitrogen source:

2.0 gm/l potassium nitrate

Test variables:

A = 2 gm/l sodium acetate
B = 2 gm/l potassium nitrate
C = 10 gm/l vitamins

Table 13: Run 1 of 5 liter fermenters testing acetate, nitrate and vitamins

Time (hr)	Fermenter	ABS_{600}	pH	Glucose gm/l	Alkaline Phosphatase units/ml
4	A	0.280	7.60	--	0.52
	B	0.309	7.57	--	0.60
	C	0.292	7.60	--	0.72
8	A	0.340	7.75	1.0	1.30
	B	0.398	7.66	1.0	1.79
	C	0.396	7.62	1.0	1.83
16	A	0.418	7.72	1.0	2.55
	B	0.462	7.51	1.0	3.72
	C	0.452	7.59	1.0	4.32
24	A	0.476	7.69	0.5	4.70
	B	0.478	7.51	0.5	7.38
	C	0.462	7.56	0.5	6.48
31	A	0.464	7.64	0.5	5.92
	B	0.498	7.47	0.25	13.68
	C	0.448	7.52	$\pm$	9.44
36	A	0.434	7.58	0.5	5.92
	B	0.488	7.43	0.25	15.68
	C	0.446	7.48	0	11.92
43	A	0.442	7.66	0.5	9.00
	B	0.480	7.51	$\pm$	20.8
	C	0.426	7.54	0	14.5
46	A	0.456	7.71	0.5	10.2
	B	0.472	7.51	$\pm$	21.7
	C	0.438	7.57	0	15.3
48	A	0.426	7.63	0.5	9.3
	B	0.450	7.45	0	20.9
	C	0.434	7.50	0	15.3

Time (hr)	Fermenter	ABS_{600}	pH	Glucose gm/l	Alkaline Phosphatase units/ml
50	A	0.446	7.76	0.5	10.7
	B	0.444	7.61	0	19.3
	C	0.410	7.65	0	15.2
52	A	0.436	7.65	0.5	10.7
	B	0.442	7.45	0	23.4
	C	0.420	7.48	0	16.3
53	A	0.444	7.61	0.5	10.3
	B	0.450	7.39	0	21.3
	C	0.424	7.44	0	15.3

Run 2:

In the second of the three examples, potassium nitrate, the additional source of nitrogen, was omitted and the test variables were as listed below. Air flow in A was partially blocked for several hours early in the run, but was fixed by 27 hrs.

Test variables:

A = 500 rpm at 0 hr; decrease to 300 rpm at 25 hrs; decrease to 200 rpm at 36 hrs.
B = 0.05 mM cobalt chloride was added at 36 hrs. 300 rpm initially was decreased to 200 rpm at 36 hrs.
C = 300 gm glass beads were placed in the bottom of the fermenter. 300 rpm initially was decreased to 200 rpm at 36 hrs.

Table 14: Run 2 of 5 liter fermenters testing agitation rate, cobalt and glass beads

Time (hr)	Fermenter	ABS_{600}	pH	Glucose gm/l	Alkaline Phosphatase units/ml
6	A		7.85		0.95
	B		7.78		1.69
	C		7.82		0.96
12	A		7.72	1	1.76
	B		7.67	1	2.34
	C		7.68	1	2.54
22	A		7.75	1	4.26
	B		7.68	1	5.70
	C		7.64	1	4.26
27	A	0.506	7.63	1	5.60
	B	0.604	7.61	1	7.32
	C	0.526	7.57	1	6.36
35	A	0.530	7.68	1	8.12
	B	0.620	7.66	0.5	8.48
	C	0.538	7.61	0.5	8.16
43	A	0.550	7.68	1	13.3
	B	0.636	7.65	0.5	13.6
	C	0.490	7.60	0.25	11.0
47	A	0.558	7.68	1	15.7
	B	0.668	7.61	0.25	14.9
	C	0.450	7.54	0	9.9
50	A	0.562	7.73	0.5	14.5
	B	0.666	7.67	$\pm$	13.9
	C	0.418	7.59	0	8.6

Run 3:

For the third of the example runs in the 5 liter fermenters, the agitation rate was started at 100 rpm and aeration at 0.25 ml air/ml medium/min. At 1 hr, rates were increased to 400 rpm and 0.5 ml air respectively. At 4 hrs, rates were further increased to 400 rpm and 1 ml air. At 24 hrs, the agitation was decreased to 300 rpm and further decreased at 35 hrs to 200 rpm. It was most unusual, but there was no visible growth in fermenter C through 14 hrs. The air flow in B was noticeably restricted by 43 hrs, and almost totally restricted by 50 hrs. The base medium was the same as in run 2. This run was to test some of the trace elements.

Test variables:

A = 0.02 gm/l ferric citrate, no magnesium, no zinc.
B = 0.005 gm/l magnesium sulfate, 0.001 gm/l zinc sulfate, no iron.
C = 0.005 gm/l manganese sulfate, 0.001 gm/l zinc sulfate, no iron, no magnesium.

Table 15: Run 3 of 5 liter fermenters testing trace elements

Time (hr)	Fermenter	ABS_{600}	pH	Glucose gm/l	Alkaline Phosphatase units/ml
9	A	0.578	7.82	1	1.18
	B	0.414	7.88	1	1.02
	C	0.048	7.99	1	0.67
24	A	0.648	7.80	0.5	2.12
	B	0.730	7.86	0.5	4.24
	C	0.345	7.92	1	2.36
30	A	0.634	7.71	0.5	3.64
	B	0.724	7.75	±	6.56
	C	0.500	7.72	1	5.00
35	A	0.616	7.64	±	6.6
	B	0.720	7.77	0	8.8
	C	0.532	7.72	0.5	9.0

Time (hr)	Fermenter	ABS_{600}	pH	Glucose gm/l	Alkaline Phosphatase units/ml
43	A	0.606	7.78	0	9.2
	B	0.712	7.91	0	9.6
	C	0.560	7.88	0.25	12.6
44	A	0.602	7.84	0	11.5
	B	0.710	7.95	0	10.9
	C	0.558	7.90	±	15.0
45	A	0.594	7.81	0	10.0
	B	0.708	7.90	0	10.6
	C	0.560	7.88	0	12.4
46	A	0.592	7.81	0	8.6
	B	0.716	7.89	0	9.5
	C	0.582	7.85	0	13.2
47	A	0.582	7.75	0	8.6
	B	0.704	7.83	0	9.8
	C	0.580	7.80	0	15.8
48	A	0.592	7.76	0	8.7
	B	0.724	7.86	0	9.6
	C	0.588	7.84	0	15.2
50	A	0.572	7.74	0	9.1
	B	0.700	7.80	0	8.1
	C	0.586	7.78	0	13.1
57	C	0.618	7.75	0	12.4
59	C	0.624	7.81	0	10.7
61	A	0.658	7.75	0	9.1
	B	0.788	7.81	0	6.8
	C	0.668	7.82	0	12.8

Regardless of the variables being tested, alkaline phosphatase activity reached a maximum between 44 and 48 hrs. It was necessary to monitor enzyme levels closely near the end of the fermentation, since they did begin to fall quite quickly after the peak was reached. The absorbance tended to decrease at about the same time as the

alkaline phosphatase reached its maximum, although sometimes the curve was too flat to be a reliable indicator.

The pH generally drifted downward, reaching the minimum between 26 and 36 hrs. The maximum pH appeared to be a fairly good predictor of maximum alkaline phosphatase levels. In about half the runs, the peak alkaline phosphatase levels occurred at the same time as the peak pH. For the remainder, the peaks were within 2 to 3 hrs of each other.

The absolute value of the pH was irrelevant, possibly offering an explanation for why pH adjustment of the medium was not beneficial. The minimum pH of some runs was well above the maximum pH of other fermentations. The absolute value was likely influenced by test parameters. However, the pattern of pH and alkaline phosphatase values had a surprising degree of correlation.

Inoculation rate:

At first, very high inoculation rates were used, up to 14%, but commonly 6-8%. Later runs produced more target enzyme. It is difficult to assess from the data collected whether the high rate of inoculation, had a significant inhibitory effect on alkaline phosphatase production, because other parameters were simultaneously being changed. Data in the following table was collected at 62 °C. The effect of the inoculation rate was also determined under a number of other temperatures. It was concluded that a 0.5% inoculum was sufficient.

Table 16: Influence of inoculation rate on alkaline phosphatase production

Inoculum (%)	Alkaline phosphatase/ml
0.5	0.351
0.5	0.379
2.0	0.273
2.0	0.183
5.0	0.266
5.0	0.213

pH:

Buffering capacity was important. Metabolic products resulted in a pH decrease, where more acid phosphatase and less alkaline phosphatase would be produced. (Nahas) It was theorized that addition of base to maintain an elevated pH would be of value although it was also known that if the pH was too high, alkaline phosphatase activity would begin to fall. For example, *Citrobacter* grown at pH 6, 7 and 8, displayed phosphatase specific activities of 1.170, 1.473 and 0.589 units/mg, respectively. (Hambling) However, as noted above, the pattern of the pH fall and rise appeared to be of far more importance than the actual pH.

Table 17: Effect of pH adjustment

Treatment	Final pH	Final alkaline phosphatase per ml
10 sucrose	7.08	17.74
10 sucrose, pH adjust 24, 33 & 46 hr	7.52	14.08

In this example, adding Tris base to raise the pH did not result in increased alkaline phosphatase production. Additional tests under other conditions (Table 11) provided similar results, indicating no correlation between alkaline phosphatase production and the final pH of the cultures.

The following table is arranged in chronological order. Note that the addition of the half salts, maintaining the original volume did increase the alkaline phosphatase production. Also the table shows a general increase in alkaline phosphatase production as various parameters were optimized. However, the level of alkaline phosphatase was not directly correlated with the final pH.

Table 18: Lack of correlation between final pH and alkaline phosphatase production

5 liter fermenter		14 liter fermenter	
Final pH	**Alk phos/ml**	**Final pH**	**Alk phos/ml**
7.57	4.58	7.68	3.74
7.67	5.14	7.84	6.68
7.42	5.96	7.43	5.75
Began addition of half salts so that the volume was maintained at the starting level.			
7.27	16.08	7.36	18.40
7.41	16.08	7.56	15.76
7.51	15.80	7.61	19.80
7.39	16.95	7.45	18.00
7.78	17.40		
7.56	25.40		
7.71	10.20		
7.51	21.70		
7.57	15.30		
7.68	15.70		
7.61	14.90		
7.54	9.90		
7.75	8.60		
7.83	9.80		
7.80	15.80		

Aeration:

Oxygen availability is determined by reactor configuration, rate of oxygen addition, temperature and oxygen use by the biomass. One form of stress reported in the literature that results in an increase in alkaline phosphatase production is low oxygenation. (Torriani) Another study looked at oxygen dependence for the expression of alkaline phosphatase activity. They systematically varied the oxygen/nitrogen ratio in the gas stream brought to equilibration in the growth medium. Under anaerobic conditions, no alkaline phosphatase was detected. The enzyme activity rose in a cooperative fashion toward a maximum activity at 14% oxygen. However, under extreme aerobic conditions, alkaline phosphatase production again fell to nondetectable levels. Although a number of regulatory genes are known to be involved in *phoA* expression, induction of activity by oxygen could be achieved by a classical allosteric protein binding directly to DNA. Decline of activity at higher oxygen concentrations, however, would require a more complex control mechanism. A similar

pattern of oxygen effects on alkaline phosphatase production by JKR209 was observed here. Very high and very low levels of oxygenation were inhibitory: intermediate oxygenation resulted in maximum alkaline phosphatase levels.

Most of these studies were performed in 500 ml minireactors. Results were confirmed in stirred bioreactors. Routinely, fermenters were aerated at 0.8-1.0 ml air/ml medium/min. First, the aeration rate was dropped to determine the effect. At the time this experiment was run, these were reasonably high levels of enzyme production.

Table 19: Effect of decreasing aeration

Fermenter	Aeration rate	Time (hr)	Abs_{600}	pH	Alkaline phosphatase per ml
5 liter	0.5 ml	44 hr	0.610	7.67	5.14
14 liter	0.3 ml	44 hr	0.430	7.68	3.74

Then the effect of increasing aeration was determined. First, pure oxygen was used at the standard aeration rate of 1 ml/ml medium/min for both fermenters. At 24 hrs, aeration was switched to 1 ml air/ml medium/min. The reasoning was that maximum aeration would be provided during cell growth. By the time alkaline phosphatase production was increasing rapidly, the amount of oxygen available was decreased. Used in this manner, oxygen had a strongly inhibitory effect on alkaline phosphatase production.

Table 20: Effect of increasing oxygen availability for first 24 hrs

Fermenter	Aeration rate	Time (hr)	Abs_{600}	pH	Alkaline phosphatase per ml
5 liter	1 ml O_2, then air	49	0.378	7.54	0.043
14 liter	1 ml O_2, then air	49	0.302	7.37	0.028

The next set of experiments compared the effects of the standard level of air with low and high levels of pure oxygen. The same inoculation procedure and medium was utilized as in the stirred fermenters but this experiment was run in triplicate in 500 ml minifermenters. It was theorized that the low oxygen would give results similar to that of the standard rate with air, since the amount of oxygen being delivered was approximately equal.

Normal air = 1 ml air/ml medium/min
Low oxygen = 0.2 ml oxygen/ml medium/min (same amount of oxygen as in 1 ml air)
High oxygen = 2 ml oxygen/ml medium/min

Table 21: Testing a range of oxygen levels

Aeration	Time (hr)	Abs_{600}	pH	Alkaline phosphatase per ml
1 ml air	3	.189	7.83	
		.200	7.83	
		.209	7.83	
0.2 ml O_2	3	.188	7.83	
		.200	7.83	
		.180	7.81	
1 ml O_2	3	.185	7.83	
		.187	7.83	
		.204	7.83	
1 ml air	18	.440	7.80	1.660
		.448	7.78	1.760
		.464	7.77	1.930
0.2 ml O_2	18	.301	7.76	1.044
		.317	7.74	0.964
		.312	7.74	0.984
1 ml O_2	18	.482	7.61	0.992
		.432	7.64	0.860
		.478	7.61	0.884
1 ml air	30	.544	7.54	2.800
		.542	7.48	2.656
		.558	7.49	2.720
0.2 ml O_2	30	.348	7.50	1.348
		.349	7.46	1.300
		.349	7.49	1.620
1 ml O_2	30	.612	7.28	1.260
		.726	7.24	1.452
		.698	7.29	1.416

Since aeration with 1 ml of either air or oxygen resulted in a lower final volume when compared with the low rate of oxygen, alkaline phosphatase levels were corrected to account for the volume difference.

Average for 1 ml air:	2.725
Average for 0.2 ml O_2:	1.423
Average for 1 ml O_2:	1.376

Average total alkaline phosphatase for 1 ml air:	2006
Average total alkaline phosphatase for 0.2 ml O_2:	1154
Average total alkaline phosphatase for 1 ml O_2:	768

Clearly, use of oxygen, even at a rate equivalent to the amount of oxygen in the standard rate of 1 ml air/ml medium/min was inhibitory.

One additional experiment was run to determine if cutting off the oxygen as early as 12 hours could help with biomass production yet not interfere with enzyme production. The same inoculation procedure and medium was utilized and again, the experiment was run in triplicate in minifermenters.

Table 22: Effect of increased oxygenation only during the first 12-24 hrs

	Treatment	Alkaline phosphatase/ml
A	1.0 ml air/ml medium/min. At 24 hrs reduce to 0.25 ml air	4.400
B	1.0 ml oxygen/ml medium/min. At 12 hrs reduce to 0.25 ml air	3.980
C	1.0 ml oxygen/ml medium/min. At 24 hrs reduce to 0.25 ml air	3.540

Although used in this manner, oxygen was not as inhibitory, there was no advantage to using oxygen for either biomass production or for enzyme production. In the stirred fermenters, it was later found that increasing the agitation rate initially during biomass production and then decreasing it as enzyme production began was useful.

Optimized media:

Alkaline phosphatase production is regulated by the level of phosphate available to the organism. In the presence of excess phosphate, little or no enzyme production occurs. Only when the phosphate level is very low, does alkaline phosphatase production reach the maximum rate. The problem is that without sufficient phosphate, biomass production is very low.

Experiments designed to work around this problem are discussed below. The solution was to produce cells for the inoculum in an enriched medium. Once sufficient biomass was obtained, the medium was centrifuged. Spent medium was discarded and the cells were used to inoculate the production medium, which was very low in phosphate. In

initial work, the cells were washed to remove traces of phosphate. However, this was later determined to be unnecessary.

Inoculum: The optimized formulation for the inoculum was 2.5 gm malt extract, 2.5 gm dextrose, 2.5 gm tryptone, 0.05 gm calcium chloride, 0.5 gm trace metals solution in 500 ml water. This was autoclaved in a minifermenter. After cooling, the pH was adjusted to 8.1 with sterile sodium phosphate dibasic. Cells were grown for 24 hrs at 65 °C, using 1.0 ml air/ml medium/min. The minifermenters were not equipped with condensers, so the final volume was usually about 400 ml. At an inoculation rate of 0.5%, this was sufficient for 80 liters. Depending on the size of the fermenters to be used, sufficient whole medium was centrifuged at 3200 rpm for 30 min and the supernatant was discarded. The cells were resuspended in sterile water and added to the fermenters at the appropriate rate.

Production medium:

14 liter fermenter	5 liter fermenter
Salts for 3000 ml in 2700 ml water	Salts for 3000 ml in 2700 ml water
Salts for 3000 ml in 2700 ml water	Salts for 500 ml in 500 ml water
Salts for 3000 ml in 2700 ml water	Metals, molasses etc. for 3500 ml in
900 ml water	300 ml water
Metals for 10,000 ml in 250 ml water	Total = 3500 ml
Molasses etc for 10,000 ml in 750 ml water	
Total = 10,000 ml	

Salts formulation:
1 gm/l ammonium sulfate
5 gm/l calcium chloride
3 gm/l Tris base
Adjust to pH 8.1 with Tris HCl before autoclaving.

Molasses etc formulation:

0.1 gm/l tryptone
10 gm/l sucrose
0.02 gm/l ferric citrate
3.0 gm/l molasses

Metals formulation:

0.005 gm/l magnesium sulfate
0.001 gm/l zinc sulfate

Half salts formulation:

0.5 gm/l ammonium sulfate
2.5 gm/l calcium chloride
1.5 gm/l Tris base
Adjust to pH 8.1 with Tris HCl before autoclaving.
Approximately 300 ml half salts per liter starting volume will be needed to maintain the volume in the fermenters.

PROCESSING

During Phase I work, separation of the cells and concentration of the spent medium was accomplished using the ultrafiltration system from Millipore called the Minitan. Although this was successful for small volumes, as scale up began producing over 10 liters at a time, the process was taking too long. Furthermore, when the enzyme was in contact with the filters for a long time, enzyme recovery decreased. However, based on Phase I work, it was also known that the alkaline phosphatase adhered very strongly to many filters, even those that poorly bound many other proteins.

The first filters tested were from Filtron. However, just a short centrifugation resulted in a significant loss of alkaline phosphatase activity.

Table 23: Loss of enzyme activity on Filtron filters

Filtron filter	Alkaline phosphatase	% enzyme recovered
Control (no filtration)	0.418	100
0.3 micron filter	0.344	82
30,000 MW cutoff filter	0.068	16
10,000 MW cutoff filter	0.089	21

A further review of other types of filters available indicated that A/G Technology Corp. (Needham, MA) produced a range of membrane cartridges for ultrafiltration and microfiltration that might be suitable. These utilize hollow fiber membranes manufactured in such a manner that they are free of macrovoids at the separating skin as well as within the supporting substructure. Macrovoids result in lower intrinsic strength, and can cause pinhole imperfections which reduce product recovery. Polysulfone, used in all A/G Technology filters is also used in some Minitan filters, one reason these were selected for testing. A series of experiments was conducted comparing the A/G Technology filters and the Minitan filters. This work is outlined below.

Table 24: Comparison of Millipore and A/G Technology filters

Process	Filter	Rate	Filter surface area
Clarification	Millipore	2.2 l/hr	0.048 sq meters
Clarification	A/G Technology	6.5 l/hr	0.140 sq meters
Concentration	Millipore	1.1 l/hr	0.048 sq meters
Concentration	A/G Technology	11.0 l/hr	0.460 sq meters

Clarification membranes from A/G were the 5-E. Concentration membranes from A/G were the 5-B. The Minitan was configured for 8 plates at 60 sq cm per plate. In the first experiment, the A/G Technology filter was tested for enzyme recovery during the desalting process. Only 4.5% of the enzyme was lost in the waste stream, roughly equivalent to what had been observed when using the Minitan system for desalting.

Then, the two systems were compared recording the volume and alkaline phosphatase levels in 4 samples: (1) the starting material which had simply been centrifuged to remove the bulk of the biomass; (2) clarified material passed through the 0.65 μm Millipore plates or 0.45 μm A/G Technology filters; (3) waste material; and (4) enzyme concentrate recovered from 10,000 MW cutoff filters from both systems.

Table 25: Comparison of enzyme recovery from two filtration systems

Identification	Volume	Alkaline phosphatase	
		Per ml	Total
Minitan			
Centrifuged	5248	11.32	59,277
Clarified	5248	12.28	64,445
Waste	5248	0.258	1,355
Concentrate	43.47	2050.	89,114
AG/Technology			
Centrifuged	5225	11.32	59,277
Clarified	5225	14.96	78,166
Waste	5225	0.163	859
Concentrate	104	1510.	157,040

Since the final volume for the A/G Technology system was more than double that of the Millipore system because of the larger holdup volume, the concentration of alkaline phosphatase in the A/G Technology system was lower, but the total amount of enzyme recovered was 76% greater.

Another set of comparison studies was conducted to confirm the initial findings.

Table 26: Further comparison of two filtration systems

Identification	Volume	Alkaline phosphatase	
		Per ml	Total
Minitan			
Whole broth	5746	15.20	87339
Clarified	5710	14.80	84508
Waste	5712	0.286	1634
Cells	NA	NA	NA
Average concentrate	54	1810.	97740

Total alkaline phosphatase in the concentrate was **112%** of the starting material, the whole fermentation broth.

Identification	Volume	Alkaline phosphatase	
		Per ml	Total
A/G Technology			
Whole broth	5747	16.30	93676
Clarified	5712	15.56	88879
Waste	5559	0.134	775
Cells	20	26.66	533
Average concentrate	131	1034.	135454

Total alkaline phosphatase in the concentrate was **145%** of the starting material, the whole fermentation broth. The A/G Technology filters were used for processing the remainder of the runs.

Processing time was significantly decreased using the A/G Technology system. For equivalent volumes, clarification was accomplished at 2.2 l/hr on the Minitan and 6.5 l/hr on the A/G system; concentration was accomplished at 1.1 l/hr on the Minitan and 11 l/hr on the A/G system. Surface area appears to be directly related to the processing time required. It is possible that enzyme binding is not that different between the two systems. However, because the enzyme is in contact with the membranes for much less time in the A/G system, recovery was improved.

PURIFICATION

Dye ligands:

Of all the separation mechanisms, affinity chromatography is considered the most specific, since it is based on the unique specificity inherent in a ligand-biomolecule

interaction. Although a powerful technique, its use is limited by the high cost of the affinity ligand and the instability of these preparations. (Narayanan)

Dye ligand chromatography is a variant of affinity chromatography in which synthetic dyes are used in place of natural substrates as immobilized ligands. Reactive dyes, mimicking biological ligands, offer a "nonspecific," low cost affinity medium with superior stability. The Phase I work testing a few dye ligands, was expanded.

All of the dye ligands tested here were bound to agarose. Table 24 shows the enzyme recovered in each of 11 elution tubes. The three tubes with the largest amount of enzyme recovered are highlighted. The dye ligands tested were Reactive red 120, Reactive yellow HE3G, Mimetic blue AP, Reactive yellow 2, Reactive blue 2, Reactive green 19, Reactive brown 10, Reactive blue 72, Reactive green 5, Reactive yellow 13, Reactive yellow 3, Reactive blue 4, and Reactive yellow 86. All were from Sigma, except the Mimetic blue AP from American International Chemical (ProMetic BioSciences, Burtonsville, MD). For simplicity, the "Reactive" designation has been dropped for the remainder of this discussion.

Table 27: Recovery of alkaline phosphatase from dye ligand columns

Packing	Alkaline phosphatase/ml in elution tubes A-K										
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Red 120	0.000	0.000	0.000	0.000	0.230	**10.400**	**15.360**	**3.140**	1.320	1.280	0.880
Green 19	0.000	0.000	0.000	0.000	1.080	**20.900**	**2.040**	**1.100**	0.540	0.680	0.564
Brown 10	0.022	0.000	0.000	0.000	0.880	**17.900**	**3.240**	**1.520**	0.840	0.864	0.688
Mimetic AP	0.000	0.016	0.000	1.224	**2.170**	**15.720**	**3.360**	1.320	1.040	0.908	0.540
Yellow HE3G	0.000	0.020	0.000	0.000	0.060	**15.760**	**3.680**	**0.860**	0.940	1.140	1.012
Blue 72	0.238	0.054	0.000	0.406	**7.980**	**10.320**	**1.560**	1.000	0.300	0.484	0.416
Yellow 2	0.046	0.328	0.000	0.080	0.270	**14.760**	**1.640**	**0.880**	1.040	1.108	0.984
Green 5	0.000	0.000	0.000	0.000	0.390	0.840	2.880	**4.580**	**4.300**	**4.880**	4.360
Blue 2	0.174	0.144	0.000	0.004	**8.150**	**3.920**	**1.500**	0.880	0.860	1.060	0.828
Yellow 3	0.780	0.336	0.010	**1.396**	**5.380**	**2.520**	0.460	0.220	0.200	0.336	0.288
Yellow 13	0.802	0.864	0.072	0.840	**3.170**	**2.040**	**1.060**	0.780	0.480	0.848	0.792
Blue 4	1.334	0.594	0.100	**2.360**	**1.360**	**1.860**	0.560	0.260	0.240	0.380	0.336
Yellow 86	1.702	0.936	0.154	**1.492**	**0.920**	**2.360**	0.580	0.560	0.220	0.272	0.180

Alkaline phosphatase levels in the three tubes with the highest recovery were added and used to create Table 28, allowing a ranking of the dye ligands for enzyme recovery.

Table 28: Ranking of dye ligands by amount of alkaline phosphatase recovered

Dye	Sum of 3 highest elution tubes	% Alkaline phosphatase recovered compared with Reactive red 120
Red 120	28.90	100
Green 19	24.04	83
Brown 10	22.66	78
Mimetic blue AP	21.25	74
Yellow HE3G	20.30	70
Blue 72	19.86	69
Yellow 2	17.28	60
Green 5	13.76	48
Blue 2	13.57	47
Yellow 3	9.30	32
Yellow 13	6.27	22
Blue 4	5.58	19
Yellow 86	4.77	17

The Yellow HE3G is not yet commercially available. It was prepared in the laboratory as follows:

1. 5 gm sepharose washed with water, 0.1 gm Procion yellow HE3G, 22 ml water. Mix and hold at room temperature for 5 min.
2. Add 2.5 ml 20% NaCl.
3. Mix gently for 30 min at 55 °C.
4. Add 0.25 gm sodium carbonate.
5. Mix gently overnight.
6. Wash with distilled water, drain.
7. Add 1 M ammonium chloride, pH 8.7 with dilute NaOH, allow to stand 1 hr.
8. Wash with 1 M NaCl.
9. Wash with 25% methanol in total of 100 ml.
10. Wash with 200 ml distilled water.
11. Equilibrate with buffer.

The Mimetic blue AP employs a dye like structure, but was designed specifically for purification of calf intestinal alkaline phosphatase. As will be seen later, Mimetic blue AP was particularly effective when it was shielded. However, the recovery from all columns, including the Mimetic blue AP, dropped significantly when shielding was employed.

Samples that had been purified through four different dye ligand columns were analyzed by capillary electrophoresis. The sample through the Brown 10 column had insufficient protein to make an accurate assessment. Small impurities were detected in

the remaining samples. Based on the number of peaks detected and the fraction of protein attributed to the impurities, it was determined that the Yellow HE3G dye ligand provided the greatest purification. Yellow HE3G is not commercially available. Red 120 produced alkaline phosphatase recovery and purity comparable to that obtained from Green 19. Since alkaline phosphatase eluted from the Red 120 columns had a smaller number of impurities, that packing was selected.

Yellow HE3G: 24.5% impurities (5 peaks detected), alkaline phosphatase = 75.5%

Red 120: 33.6% impurities (6 peaks detected), alkaline phosphatase = 66.4%

Green 19: 33.3% impurities (9 peaks detected), alkaline phosphatase = 66.7%

It should be noted that the capillary electrophoresis is more sensitive than the standard electrophoresis used to determine purity during Phase I.

Scale up of purification using Reactive red 120:

A number of purification experiments were set up to determine the effect of various parameters. Table 29 is a summary of results from 25 columns, arranged in order of amount of enzyme loaded on each column. Other parameters were varied, accounting for the variation at each loading rate. It can be seen, however, that loading 60,000 units or more on a column resulted in overloading and poor recovery. Optimizing other parameters, good recovery (68%) was obtained when loading as much as 22,000 units. The best recovery (81%) was from a column loaded with only 1500 units. It may be noted that if other parameters were not optimized, recovery was still low, even when the column was not overloaded.

Table 29: Purification of alkaline phosphatase in 27 mm diameter Reactive Red 120 columns

Total alkaline phosphatase loaded	Total alkaline phosphatase recovered	% alkaline phosphatase recovered	Maximum alkaline phosphatase level/ml
157,040	5,226	3	52
135,454	14,276	10	72
97,740	6,108	6	15
94,096	2,263	2	85
89,114	2,222	2	31
60,200	3,307	5	88
29,274	12,165	42	366
21,960	14,940	68	444
16,508	6,618	40	164
14,800	2,648	18	43
12,375	6,619	53	185
11,840	2,216	19	43
11,840	2,170	18	39
11,840	2,709	23	60
11,840	2,047	17	25
11,840	2,045	17	35
3,276	312	9	2
2,960	602	20	9
2,960	663	22	10
1,500	790	53	428
1,500	1,221	81	677
1,250	400	32	384
1,150	349	30	307
Second pass:			
2,844	3,051	107	67
Third pass:			
3,051	2,042	67	45

Elution was accomplished with 0.8 M calcium chloride rather than 0.6 M calcium chloride for experiments where enzyme was loaded on the same column a second or third time and for some of the first passes. Although the recovery was significantly enhanced when the higher concentration of salt was employed, the degree of purification was significantly less. A second band was observed on the electrophoresis gels.

45 ml Reactive red 120 dye columns (duplicates) were reactivated with 8 M urea in 0.5 M NaOH. Columns were then washed extensively with water followed by 20 mM Tris, pH 8.5. 15 ml of concentrated crude alkaline phosphatase, diluted with 30 ml Tris buffer, was loaded onto each column. 20 ml was discarded as waste, before starting the scheme outlined below. Values are reported as averages of the two columns loaded with identical samples.

Table 30: Scale up purification of alkaline phosphatase using Reactive red 120

Tube	Description	Alkaline phosphatase/ml
1	8 ml void	0.329
2	15 ml sample	0.158
3	8 ml sample	0.090
4	125 ml buffer	8.396
5	125 ml buffer	5.458
6	.05 $CaCl_2$ 125 ml in buffer	2.742
7	.6 $CaCl_2$ in 15 ml buffer	0.545
8	.6 $CaCl_2$ in 15 ml buffer	0.163
9	.6 $CaCl_2$ in 15 ml buffer	11.725
10	.6 $CaCl_2$ in 15 ml buffer	185.500
11	.6 $CaCl_2$ in 15 ml buffer	117.000
12	.6 $CaCl_2$ in 15 ml buffer	49.400
13	.6 $CaCl_2$ in 15 ml buffer	13.850
14	.6 $CaCl_2$ in 15 ml buffer	11.000
15	.6 $CaCl_2$ in 15 ml buffer	8.650
16	.6 $CaCl_2$ in 15 ml buffer	7.060
17	.6 $CaCl_2$ in 15 ml buffer	4.010
18	.6 $CaCl_2$ in 15 ml buffer	3.900
19	.6 $CaCl_2$ in 15 ml buffer	3.560
20	.6 $CaCl_2$ in 15 ml buffer	3.100
21	.6 $CaCl_2$ in 15 ml buffer	2.730
22	.6 $CaCl_2$ in 15 ml buffer	2.370

TOTAL = 6618.681 X 2 columns = 13,237.362

The original concentrate contained 24,750 units of alkaline phosphatase per ml so 53.5% of the enzyme loaded on the columns was recovered.

Tube 10 had 42.0% of the eluted alkaline phosphatase, tube 11 had 26.5% and tube 12 had 11.2%, so tubes 10, 11 and 12 all together have 80% of all the alkaline phosphatase eluted.

In the next experiment reported here, a total of 66,030 units of alkaline phosphatase was divided among the 4 columns, with 23.25 ml of concentrate loaded onto each

column. These were four 95 X 27 mm columns of Reactive Red 120. After being regenerated with alkaline urea, the columns were washed, loaded and eluted according to the following schedule:

A - 83.25 ml buffer/column
B - 83 ml buffer/column
C - 76.75 ml buffer/column
D - 80.25 ml buffer with 0.05 M calcium chloride/column
E-V - 10 ml of 0.6 M calcium chloride/column

The original concentrate contained a total of 66,030 units (or 16,508/column). The 40% recovery, somewhat lower than for the results reported in the previous table, may be a reflection of column overloading.

Table 31: A second example of scale up purification using Reactive red 120

Sample	Alkaline phosphatase/ml (average of 4 columns)
A	0.482
B	0.158
C	0.060
D	0.002
E	0.000
F	0.000
G	0.160
H	164.000
I	162.000
J	111.500
K	57.400
L	50.800
M	32.400
N	21.300
O	15.600
P	12.100
Q	7.520
R	6.000
S	5.920
T	5.220
U	5.020
V	4.240

Effect of metals and alternative buffer:

Since alkaline phosphatase is a known metalloenzyme, with at least 2 metal binding sites, metals were added to buffer in an attempt to enhance binding to the dye ligand.

Reactive red 120 on agarose was packed into 1 cm columns to 10-11 cm height. Following regeneration of the columns with 8 M urea in 0.5 M NaOH, they were washed extensively with water and then with buffer. The Tris buffer included the noted levels of zinc sulfate, magnesium sulfate or calcium acetate. Alternatively, a HEPES-NaOH buffer was tested. Calcium acetate was substituted for calcium chloride as noted. Appropriate buffer refers to Tris buffer with zinc or magnesium sulfate at the indicated levels or HEPES-NaOH buffer.

A 1 ml appropriate buffer + 0.5 ml concentrated sample.
B 10 ml appropriate buffer.
C 10 ml appropriate buffer.
D 10 ml appropriate buffer.
E 10 ml appropriate buffer with 0.05 M calcium chloride (acetate).
F 10 ml appropriate buffer with 0.05 M calcium chloride (acetate).
G 10 ml appropriate buffer with 0.5 M calcium chloride (acetate).
H 10 ml appropriate buffer with 0.5 M calcium chloride (acetate).
I 10 ml appropriate buffer with 0.5 M calcium chloride (acetate).

Table 32: Effect of zinc, magnesium, calcium acetate and an alternative buffer

	0.2 mM $ZnSO_4$	**2 mM $ZnSO_4$**	**1 mM $MgSO_4$**	**10 mM $MgSO_4$**	**Calcium acetate**	**HEPES NaOH**
A	0.688	0.680	0.212	0.164	0.376	0.200
B	0.600	0.416	0.648	0.348	0.896	1.184
C	0.588	0.384	0.56	1.148	0.736	1.176
D	0.352	0.392	0.616	1.020	0.452	1.368
E	0.236	0.224	0.408	0.416	0.268	-----
F	1.060	0.440	0.380	0.372	0.348	-----
G	**384.0**	**344.0**	**358.0**	**376.0**	**342.0**	**312.0**
H	4.28	33.4	36.0	46.4	32.4	44.6
I	7.8	6.8	8.08	6.24	7.08	

Alkaline phosphatase recovery in elution tube G (highest recovery) was calculated as a percentage of the maximum for each treatment and is listed in Table 33.

Table 33: Percentage of alkaline phosphatase recovered, using the maximum recovery as 100%

0.2 mM $ZnSO_4$	2 mM $ZnSO_4$	1 mM $MgSO_4$	10 mM $MgSO_4$	Calcium acetate	HEPES NaOH
100%	90%	93%	98%	89%	81%

Further runs set up in a similar fashion indicated that 0.1 mM zinc chloride and 10 mM magnesium chloride mixed was the optimum combination of metals.

Shielding chromatography:

In dye ligand chromatography, sample components are held on the dye ligand medium by both specific and nonspecific binding. In an optimized system, strong, specific binding and weak nonspecific binding hold target molecules to the dye ligand. However, contaminants are also held by the weaker but more numerous nonspecific bonds.

Although dye ligand chromatography achieved remarkable purification of the alkaline phosphatase studied here, the separation was still plagued by two problems. (1) One was that binding of the alkaline phosphatase was so strong that complete elution was not achieved. Harsh conditions required resulted in elution of additional contaminants. (2) The second problem was that the weak, nonspecific binding of contaminants compromised the purification protocol, requiring additional purification steps.

Shielding chromatography was examined as a possible solution to this dilemma. By covering the dye ligand matrix with a protective polymer coating, it was theorized that the strong specific binding would be reduced and the weak nonspecific binding would be eliminated. (Galaev) This would eliminate the necessity of using very strong calcium chloride solutions for elution and would eliminate binding of the contaminants.

Columns were regenerated with 8 M urea in 0.5 N NaOH. Following an extensive wash with water, columns were shielded by treating them with 1% polyvinylpyrrolidone (PVP), a nonionic polymer. This was followed by a wash with 1.5 M KCl, pH 3.4 (Tris HCl + HCl) and a water wash. Columns were equilibrated with 20 mM Tris buffer.

0.1 ml concentrated alkaline phosphatase was loaded onto each column. Tubes B and C represent buffer washes of 10 ml each. Tube D is from another 10 ml buffer wash but with 0.05 M calcium chloride added. Tubes E-K are the 2.5 ml of 0.6 M calcium chloride in buffer used to elute the enzyme.

Table 34: Shielded dye ligand columns for alkaline phosphatase purification

PACKING	A	B	C	D	E	F	G	H	I	J	K
Mimetic AP	0.042	0.086	0.030	0.094	**3.040**	**9.120**	1.900	1.080	0.386	1.168	0.728
Brown 10	0.792	0.564	0.078	0.896	**4.520**	**3.980**	0.580	0.040	0.202	0.164	0.172
Green 5	1.416	0.542	0.028	1.132	**3.580**	**2.620**	0.660	0.280	0.488	0.354	0.362
Yellow 2	0.634	0.700	0.078	**1.168**	**1.080**	1.000	0.480	0.420	0.688	0.376	0.468
Red 120	0.444	0.596	0.082	0.844	**1.020**	**1.060**	0.260	0.180	0.470	0.388	0.416
Blue 72	1.436	1.052	0.280	**1.076**	**0.780**	1.020	0.180	0.080	0.210	0.184	0.220
Yellow HE3G	0.984	0.812	0.140	0.582	**0.800**	**0.800**	0.300	0.300	0.484	0.426	0.520
Green 19	2.260	0.928	0.056	0.338	**0.420**	**0.440**	0.100	0.080	0.218	0.196	0.246

Since the Mimetic blue AP was designed specifically for alkaline phosphatase, it would be expected that the binding was specific. For dye ligands, it is assumed that the binding is a combination of specific and nonspecific. This reasoning was supported by the results of the shielding chromatography. The shielding would largely cover nonspecific binding sites, leaving only those packings with specific binding to effectively bind the alkaline phosphatase. In this case, Mimetic blue AP performed significantly better than any other dye ligand column, likely because the enzyme bound poorly to the largely covered nonspecific sites in the standard dye ligand columns, but bound well to the specific sites of the Mimetic blue AP.

Four more trials with shielded dye ligands were run. Some, including the results in Table 34 were done in 9 mm columns. Others were scaled up to 27 mm columns. Averages for 7 packings taken from 3-5 runs were calculated and the packings ranked for the amount of alkaline phosphatase recovered, using the Reactive red 120 as the standard.

It appeared that the Yellow HE3G prepared at J. K. Research did not adhere as well to the agarose as dye ligands that were commercially available. New columns bound alkaline phosphatase very well but following reactivation with the alkaline urea, binding efficiency dropped significantly.

Table 35: Efficacy of shielded dye ligand columns for alkaline phosphatase purification

Dye	%Alkaline phosphatase recovered compared with Red 120
Mimetic blue AP	284
Brown 10	153
Yellow HE3G without urea	130
Red 120	100
Yellow 2	91
Green 19	84
Blue 72	69
Yellow HE3G with urea	52

Optimized use of dye ligand columns:

Following is the method used for dye ligand columns for alkaline phosphatase.

Column regeneration:

8 M urea in 0.5 M NaOH, 2 column volumes
Wash extensively with distilled water

Purification:

Load sample onto column diluted in an equal volume of column buffer (20 mM Tris, 10 mM magnesium sulfate, 0.1 mM zinc sulfate. Adjust to pH 8.6 with 1 N HCl).
Wash with 3 column volumes of column buffer.
Wash with 1 column volume of 20 mM Tris, pH 8.6 (no metals) + 0.05 M calcium chloride.
Elute with 10 ml aliquots of 20 mM Tris (no metals) + 0.6 M calcium chloride.

ALTERNATIVE CHROMATOGRAPHIC METHODS

Histidyldiazobenzylphosphonic acid packing:

Histidyldiazobenzylphosphonic agarose columns have been used for the purification of alkaline phosphatase. Enzyme from *Thermotoga neopolitana* was purified 2880 fold with a 45% yield. However, this could only be accomplished in the presence of cobalt. (Dong) This same purification procedure was attempted for the alkaline phosphatase from JKR209.

Alkaline phosphatase concentrate (0.2 ml) was loaded onto an L-histidyldiazobenzylphosphonic acid column. It was washed with buffer and buffer with 0.05 M calcium chloride. Elution was accomplished with 2 ml aliquots of 0.6 M calcium chloride. Results were compared with a Reactive red 120 column for efficiency of recovery. Significantly more enzyme was recovered from the L-histidyldiazobenzylphosphonic acid column but in the initial trial, the elution peak was very broad. Addition of cobalt did not result in significant improvement, and use of this column was not pursued.

Substrate elution:

To increase specificity of elution, it was decided to test the efficacy of alkaline phosphatase elution by its substrate, phosphate. Following loading and washing by the standard procedure, alkaline phosphatase was eluted with 10 mM sodium phosphate in column buffer. The problem with this approach is that the substrate must then be removed before the enzyme activity can be quantitatively determined. Eluate was placed in a 1000 MW cutoff dialysis tube and dialyzed against water for 3 days.

After dialysis, significant levels of alkaline phosphatase could be detected. However, the method was very inefficient and no further attempts to use this method were made.

Size exclusion chromatography:

Following passage through the red 120 column, samples were largely purified, up to 90%. To remove the remaining contaminants, alternative methods were examined. Size exclusion chromatography was performed using Sephacryl S-100 as the column packing. Buffer composition was 20 mM Tris, 10 mM magnesium chloride, 50 mM $CaCl_2$ $2H_2O$, pH 8.5. Protein was measured as absorbance at 280 nm. This method could remove all contaminating bands observed by standard electrophoretic methods. Results recorded in the following table are from duplicate runs, recorded separately.

Table 36: Protein yield and enzymatic activity SEC = Size exclusion chromatography

Packing	**Vol ml**	**Protein mg/ml**	**Total protein**	**Activity**	**Total activity**	**Specific activity**	**Yield %**	**Purification factor**
Red 120	13.0	4.7	61.7	0.83	0.83	10.8	100	1
SEC	3.2	10.0	31.8	2.7	2.7	8.6	80	1.5
Red 120	11.5	8.8	100.0	2.0	23.1	0.23	100	1
SEC	3.0	19.5	59.0	3.1	9.2	0.16	40	0.7

Reversed phase HPLC:

For most applications, use of the Red 120 column followed by size exclusion chromatography, resulted in a single band upon electrophoresis. However, for some of the molecular biology work, it was necessary to perform one additional purification step. Reversed phase HPLC was selected and was performed in 0.1% TFA buffer. A gradient of TFA and acetonitrile served as the mobile phase. A C-8 column was used.

Capillary electrophoresis:

Although capillary electrophoresis confirmed the purity of the alkaline phosphatase fraction from the size exclusion column, it was necessary to remove small artifacts by reverse phase HPLC before N-terminal sequencing could be successfully performed. The enzyme was purified on two occasions and their molecular weights, as determined by capillary electrophoresis, matched closely: 40,437$\pm$ 614 Da and 42,701 $\pm$1055 Da This was performed on a Biorad unit, using SDS and the capillary zone exclusion.

APPLICATIONS

Heavy metal removal from waste water:

Numerous studies have shown the value of using microorganisms to remove heavy metals from water. The one major disadvantage with biological processes is that the microorganisms can only tolerate up to about 10 mg/l of chromium, copper, nickel and zinc before their effectiveness begins to decline. Mullen et. al. removed 12% of cadmium from a 1 mM solution (approximately 112 mg/l) (Mullen). Macaskie's group, using immobilized cells could remove as much as 94% of cadmium from a 200 mg/l solution passed over the cells at 200 ml/hr. As the flow rate was increased to 500 ml/hr, the removal rate dropped to 20% for cadmium. 90% of copper could be removed from a 5 mg/l solution but as copper levels were increased to 50 mg/l, the copper removal decreased to 30% (Peters).

It has been determined that insoluble metal phosphates are formed, precipitating out of solution. (Macaskie) The general procedure was to mix a source of phosphate, a metal in soluble form and alkaline phosphatase. As the enzyme released phosphate from the phosphate complex, metal phosphates were formed and precipitated.

It has also been previously established that phosphate must be in an organic form. An inorganic phosphate source was tested and resulted in no formation of metal phosphates. Glycerol-2-phosphate, an organic source of phosphate, was used for all the experiments reported here.

Experiments tabulated in the next 5 tables were always compared with a negative

control to which no alkaline phosphatase had been added. The calculation of the percentage of metal removed used this control tube as containing 100% of the starting level, or zero percent removed.

Time: Time allowed for the reaction to take place, directly affected the amount of metal removed. For example, 1 mM glycerol-2-phosphate, 1 mM cadmium and 0.5 ml alkaline phosphatase resulted in removal of 72.1% of the cadmium after standing for 5 min. Allowing the same reaction to proceed over 5 hrs, resulted in removal of 92.3% of the cadmium.

Phosphate level: It was found that the amount of phosphate affected the efficacy of the metals removal. Interestingly, too much phosphate gave poor results. As the rate of phosphate added was decreased from 10 to 1 to 0.1 mM (as phosphorus), the amount of cadmium removed increased from 27.9 to 92.3 to 94.3%.

Table 37: Effect of variation in phosphate level on metals removal

	mg/l	**% removed**
1 mM P 1 mM Cd	95.2	0.0
10 mM P 1 mM Cd 0.1 ml alkaline phosphatase	68.6	27.9
1 mM P 1 mM Cd 0.1 ml alkaline phosphatase	7.32	92.3
0.1 mM P 1 mM Cd 0.1 ml alkaline phosphatase	5.40	94.3

Alkaline phosphatase level: Rather surprising, considering the results of the previous table, it was found that addition of more alkaline phosphatase increased the amount of cadmium removed. As alkaline phosphatase rates increased from 1 µl to 1 ml (in a total volume of 10 ml), the amount of cadmium removed increased from 39.8% to 98.9%.

Table 38: Effect of rate of use of alkaline phosphatase

	mg/l	% removed
1 mM P 1 mM Cd	95.2	0.0
1 mM P 1 mM Cd **1 µl alkaline phosphatase**	57.3	39.8
1 mM P 1 mM Cd **10 µl alkaline phosphatase**	25.6	72.1
1 mM P 1 mM Cd **0.1 ml alkaline phosphatase**	7.32	92.3
1 mM P 1 mM Cd **1 ml alkaline phosphatase**	1.00	98.9

Heavy metal under test: Some heavy metals were much more amenable to removal in this system than others. Lead, copper and cadmium proved to be the easiest to remove from solution, while chromium proved to be the most recalcitrant. One suggestion at this point is to test the value of adding chromium reductase before treatment or as an alternative treatment. Chromium reductase converts highly toxic chromium VI to nearly non toxic chromium III. Several organisms in the J. K. Research collection are known to produce chromium reductase.

Table 39: Heavy metals tested in metal-phosphate system

	mg/l	% removed
Cadmium		
Control No alkaline phosphatase	95.2	
1 mM P 1 mM Cd 0.1 ml alkaline phosphatase	7.32	92.3
Chromium		
Control No alkaline phosphatase	138	
1 mM P 1 mM Cr 0.1 ml alkaline phosphatase	125	9.4
Copper		
Control No alkaline phosphatase	48.6	
1 mM P 1 mM Cu 0.1 ml alkaline phosphatase	2.45	95.0
Lead		
Control No alkaline phosphatase	2.70	
1 mM P 1 mM Pb 0.1 ml alkaline phosphatase	<0.05	>99.98
Nickel		
Control No alkaline phosphatase	136	
1 mM P 1 mM Ni 0.1 ml alkaline phosphatase	96.7	28.9

	mg/l	% removed
Zinc		
Control	198	
No alkaline phosphatase		
1 mM P	67.4	66.0
1 mM Zn		
0.1 ml alkaline phosphatase		

Concentration of metal: Perhaps the biggest advantage of this method for heavy metal removal is that it works well at high concentrations of metals. Processes utilizing whole cells for metals removal can tolerate only up to 10 mg/l of many toxic metals. This process actually increased in efficiency when as much as 1100 mg/l of metals were tested.

Table 40: More efficient removal of high concentrations of metals (% removed)

Metal	High level up to 1100 mg/l	Low level up to 200 mg/l	Low level by Macaskie
Cadmium	95.5	92.3	90
Chromium	59.5	9.4	32
Copper	91.7	95.0	97
Lead	99.8	99.98	97
Nickel	72.9	28.9	
Zinc	97.2	66.0	

Temperature: As the temperature increased, the amount of zinc removed from solution also increased. It is possible that this is simply a matter of increasing the reaction rate. However, the increased temperature appeared to increase the amount of zinc removed from the control as well as the test, so it is difficult to interpret the data. The short incubation time may have influenced the results.

100 µl zinc sulfate, 100 µl phosphate, 100 µl alkaline phosphatase, and 9.7 ml Tris buffer, pH 9, were mixed after being prewarmed separately. Following a 20 min reaction, the tubes were placed in an ice bath. Samples were centrifuged for 30 min at 3200 rpm.

Table 41: Temperature effects on efficiency of zinc removal

Temperature	Test	Control	% removed
20 °C	15.1	16.6	9.0
60 °C	12.3	16.7	26.4

pH effects: In most metal removal processes, pH often plays a crucial role in the efficiency of the method. The same applied to the formation of metal phosphates. It was anticipated that the optimum pH for metals removal would correspond to the optimum pH for the alkaline phosphatase. That did not prove to be true. Although the optimum pH for activity of this alkaline phosphatase is approximately 9.8, zinc phosphate was formed at much higher levels at pH 7 as may be seen in the following table.

100 µl zinc sulfate, 100 µl phosphate, 100 µl alkaline phosphatase, and 9.7 ml 40 mM Tris buffer, adjusted to pH 7, 9 or 11 with dilute HCl were mixed and allowed to react for 48 hrs. Tubes were not centrifuged.

Table 42: pH effects on removal of zinc from solution

	Test	Control	% removed
pH 7	28.4	167	83.0
pH 9	14.5	27.1	46.5
pH 11	20.2	28.6	29.4

Uses of metal phosphates: Many metal phosphates have a viable commercial market so could be used as produced. Alternatively, precipitates could be resolubilized and converted to other metal complexes. Following are some applications (Budavari).

Chromic phosphate: Green pigment, in wash primers, in catalysts for dehydrogenation of hydrocarbons or polymerization of olefins.

Nickel phosphate: On ignition yields "nickel yellow," a pigment in oil and water colors.

Lead phosphate: Stabilizer for plastics.

Cupric phosphate: Fungicide, fertilizer, catalyst, emulsifier, and protectant for metal surfaces against oxidation.

Zinc phosphate: In dental cement.

Bleaching agent:

Peroxymonophosphates are known to be useful in germicidal, bleaching, dyeing and metal detoxication processes. (Castrantas) A 1972 patent described the multiple uses. FMC Corporation had the capability of producing large quantities of peroxydiphosphate, the starting material. The process involved adding alkaline phosphatase or acid phosphatase, depending on the application, to peroxydiphosphate, allowing the phosphatase to generate peroxymonophosphate at a controlled rate.

At the time of the patent preparation, alkaline phosphatase was obtained largely from calf intestine. Lack of stability of the enzyme precluded its application in several industrial settings, and the process was never widely applied. With the availability of an extremely heat stable alkaline phosphatase, the process again becomes extremely attractive.

Materials stained with either coffee or tea were tested. As a positive control, 0.5 ml bleach (sodium hypochlorite) was added diluted with 40 ml water. Distilled water served as the negative control. The test solution included 100 µl alkaline phosphatase concentrate, 0.5 gm peroxydiphosphate and 40 ml water. The stained materials were allowed to dry overnight. Identical pieces cut from the same piece of material were soaked in each of the three solutions. Both the bleach and the peroxymonophosphate could bleach the materials. Although the sodium hypochlorite worked faster, the advantage of the peroxymonophosphate is that chlorine is not produced.

Chemical agent degradation:

Alkaline phosphatase has been tested in a GD and VX degradation scheme. (Dutch) The enzyme accelerated hydrolysis of both agents. A crude preparation of JKR209 alkaline phosphatase was tested in the laboratory of Dr. Joe DeFrank for degradation of organophosphorus nerve agents. It was not found to have any advantage over the DFPase, but there is still the possibility that it would be useful to degrade initial breakdown products. The crude preparation caused to much interference on the HPLC scan. A partially purified preparation should be tested when it is available in sufficient quantity.

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